DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE SIMULTANEOUS ESTIMATION OF TELMISARTAN AND CHLORTHALIDONE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM

Dissertation Submitted to

The Tamil Nadu Dr.M.G.R. Medical University, Chennai – 600 032.

In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

PRABANANTHAN A

Register No. 26116122

Under the Guidance of

Mr. K. ANANDAKUMAR M.Pharm.,

Associate Professor.

(Department of Pharmaceutical Analysis)

ADHIPARASAKTHI COLLEGE OF PHARMACY

(Accredited by "NAAC" with CGPA of 2.74 on a four point scale at "B" Grade)

MELMARUVATHUR – 603 319

APRIL – 2013

CERTIFICATE

This is to certify that the research work entitled **"DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE SIMULTANEOUS ESTIMATION OF TELMISARTAN AND CHLORTHALIDONE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM"** submitted to The Tamil Nadu Dr. M.G.R. Medical University in partial fulfillment for the award of the Degree of Master of Pharmacy (Pharmaceutical Analysis) was carried out by **A.PRABANANTHAN (Register No. 26116122)** in the Department of Pharmaceutical Analysis under my direct guidance and supervision during the academic year 2012-2013.

Place: Melmaruvathur
Date:

Mr. K. ANANDAKUMAR, M.Pharm.,
Associate Professor,
Department of Pharmaceutical Analysis,
Adhiparasakthi College of Pharmacy,
Melmaruvathur – 603 319.

CERTIFICATE

This is to certify that the research work entitled **"DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE SIMULTANEOUS ESTIMATION OF TELMISARTAN AND CHLORTHALIDONE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM"** submitted to The Tamil Nadu Dr. M.G.R Medical University in partial fulfillment for the award of the Degree of Master of Pharmacy (Pharmaceutical Analysis) was carried out by **A.PRABANANTHAN (Register No. 26116122)** in the Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, Melmaruvathur which is affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, under the guidance of **Mr. K. ANANDAKUMAR, M.Pharm.,** Associate Professor, Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, during the academic year 2012-2013.

Place: Melmaruvathur

Date :

Prof. (Dr.) T. VETRICHELVAN, M.Pharm., Ph.D.,

Principal& Head,

Adhiparasakthi College of Pharmacy,

Melmaruvathur – 603 319.

Dedicated

To

My beloved Parents

ACKNOWLEDGEMENT

First and foremost I wish to express my deep sense of gratitude to His Holiness **Arul Thiru Amma** President, ACMEC Trust, Melmaruvathur. For his ever blessing in each step of study and I am grateful to **Thirumathi Lakshmi Bangaru Adigalar**, Vice President, ACMEC trust, Melmaruvathur for having given me an opportunity and encouragement all the way in completing the study.

With great respect and honor, I extend my thanks to our Correspondent **Mrs. B. Umadevi, M. Pharm.,** Adhiparasakthi Institute of Paramedical Sciences, Melmaruvathur for her excellence in providing skillful and compassionate spirit of unstinted support to our department for carrying out the research work.

I received initiation and inspiration to undergo experimental investigation of modern analytical methods entitled as **"Development and Validation of Analytical Methods for the Simultaneous Estimation of Telmisartan and Chlorthalidone in Bulk and in Pharmaceutical Dosage Form"** I got inward bound and brainwave to this extent, I concede my inmost special gratitude and thanks to my guide **Mr. K. Anandakumar**, **M.Pharm.,** Associate Professor, Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, for the active guidance, infinite helps, indulgent and enthusiastic guidance, valuable suggestions, where the real treasure of my work. This thesis acquires the present shape due to his full concentration and help. Once again I place on record my deep sense of gratitude to him.

I take this opportunity to express my sincere thanks to our respected Principal **Prof. (Dr.) T. Vetrichelvan, M.Pharm., Ph.D.,** Principal and Head for his constant enduring support and encouragement. Without his supervision it would have been absolutely impossible to bring out the work in this manner.

I have great pleasure in express my sincere heartfelt thanks to **Dr. D. Nagavalli, M. Pharm., Ph.D.,** Professor and **Mrs. G**. **Abirami**, **M. Pharm.,** and **J. Saminathan, M.Pharm.,** Assistant Professors, Department of Pharmaceutical Analysis, for their encouragement and support for the successful completion of this work.

I have great pleasure in express my sincere heartfelt thanks to **Mr. M. Jegadeeshwaran M.Pharm.,** Assistant Professor, Nandha college of Pharmacy Erode and **Mr. J. Ramesh M.Pharm.,** Assistant Professor, JKK Muniraja College of Pharmacy, Kumarapalayam for have timely helped during my project.

My special thanks to our librarian **Mr. M. Suresh, M.L.I.S**., for providing all reference books to make this project a great success.

My genuine thanks to **Mr. R. Sankar B.Pharm., Mr. S. Selvam B.Pharm., Miss. S. vijayashanthi M.Pharm., Mr. R. Hemachandhar M.Pharm.,** and **Mr. Kamaraj M.Pharm.,** and for have timely helped during my project.

I would be failing in my duties if I do not thank my beloved classmates **Miss. P. Veerasundhari, Miss. Aruna Teja Muvva,** and **Miss. Ramya Sai Talam** for their constant support in every endeavor of mine and provided me with necessary stimulus for keeping the driving force integrated for successful completion of this project.

My special thanks to my ever loving **College staffs, seniors and juniors** for their kind support during my work.

I will never forget the care and affection bestowed upon me by my friends **S. Nandha kumar, L. Lakshmikanth, D. Bharathiraja, J. Srikanth, B. Sakthivel, B. Raghu ram, P. Niranjan, S.R.V. Vivek, K. Nagaraju** and **All My Friends** for encouraged till the day.

Special thanks to **My Child hood Teachers,** and **APEX Company friends** for their valuable support and sparing their knowledge.

Last but not least, with my whole heart, I would like to express my love to my Father **Mr. K. Appadurai,** my Mother **Mrs. A. Chithiraiselvi**, my Brother **Mr. A. Soundharrajan D.Vis.Com.,** and my relatives for their encouragement and support which were a tower of strength during the entire course of work.

Above all I dedicate myself and my work represented to **Almighty,** I stand in front of almighty with folded hands to say **"Thanks for everything"**

A.PRABANANTHAN

CONTENTS

SECTION	TITLE	Page No.
1.	**INTRODUCTION**	1
	1.1 Introduction to Analytical Chemistry	1
	1.2 Aim & Objective	1
	1.3 Analytical Methods	1
	1.4 Analytical Techniques	2
	1.5 Spectroscopic Method	2
	1.6 Chromatographic Methods	26
	1.7 Analytical Validation	46
	1.8 Basic Statistical Parameters	53
	1.9 Quality & analytical Problem	55
	1.10 Analytical Errors	56
2.	**LITERATURE REVIEW**	58-74
	2.1 Drug Profile	58
	2.2 Reported Methods	65
3.	**AIM AND PLAN OF WORK**	75-77
	3.1 Aim of Work	75
	3.2 Plan of Work	75
4.	**MATERIALS AND METHODS**	78-94
	4.1 Materials	78
	4.2 Methods	82
	4.2.1 UV Spectroscopy	82
	4.2.2 HPLC	85
	4.2.3 HPTLC	91
5.	**RESULTS AND DISCUSSION**	95-100
	5.1 UV Spectroscopy	95
	5.3 HPLC	97
	5.2 HPTLC	99
6.	**SUMMARY AND CONCLUSION**	101-169
	6.1 UV Spectroscopy	101
	6.2 HPLC	102
	6.3 HPTLC	103
7.	**BIBLIOGRAPHY**	170

LIST OF FIGURES

FIGURE No.	SUBJECT
1	IR SPECTRUM OF TELMISARTAN
2	IR SPECTRUM OF CHLORTHALIDONE
3	OVERLAIN ZERO ORDER SPECTRA OF TELMISARTAN AND CHLORTHALIDONE
4	OVERLAIN FIRST ORDER DERIVATIVE SPECTRUM OF TELMISARTAN AND CHLORTHALIDONE
5	CALIBRATION GRAPH FOR CHLORTHALIDONE AT 251nm (FIRST ORDER DERIVATIVE METHOD)
6	CALIBRATION GRAPH FOR TELMISARTAN AT 311nm (FIRST ORDER DERIVATIVE METHOD)
7	OVERLAIN SPECTRA OF TELMISARTAN AND CHLORTHALIDONE (DETECTION WAVELENGTH)
8	INTIAL SEPARATION CONDITIONS IN ACETONITRILE: METHANOL (50:50 % v/v)
9	INTIAL SEPARATION CONDITIONS IN ACETONITRILE: AMMONIUM ACETATE BUFFER (pH 5) (50:50 % v/v)
10	INTIAL SEPARATION CONDITIONS IN ACETONITRILE: PHOSPHATE BUFFER (pH 3.5) (50:50 %v/v)
11	INTIAL SEPARATION CONDITIONS IN ACETONITRILE: PHOSPHATE BUFFER (pH 3.5) (70:30 %v/v)
12	OPTIMIZED CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE
13	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE, (5, 2 µg/ ml)
14	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (10, 4µg/ ml)
15	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (15, 8µg/ ml)
16	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (20, 12µg/ ml)
17	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (25, 16µg/ ml)

18	CALIBRATION CURVE FOR TELMISARTAN BY RP – HPLC
19	CALIBRATION CURVE FOR CHLORTHALIDONE BY RP – HPLC
20	CHROMATOGRAM FOR ANALYSIS OF FORMULATION ERITEL CH 40 REPEATABILITY – 1
21	CHROMATOGRAM FOR ANALYSIS OF FORMULATION ERITEL CH 40 REPEATABILITY – 2
22	CHROMATOGRAM FOR ANALYSIS OF FORMULATION ERITEL CH 40 REPEATABILITY – 3
23	CHROMATOGRAM FOR ANALYSIS OF FORMULATION ERITEL CH 40 REPEATABILITY – 4
24	CHROMATOGRAM FOR ANALYSIS OF FORMULATION ERITEL CH 40 REPEATABILITY – 5
25	CHROMATOGRAM FOR ANALYSIS OF FORMULATION ERITEL CH 40 REPEATABILITY – 6
26	CHROMATOGRAM FOR FIRST RECOVERY OF FORMULATION – 1 (ERITEL CH- 40)
27	CHROMATOGRAM FOR FIRST RECOVERY OF FORMULATION – 2 (ERITEL CH- 40)
28	CHROMATOGRAM FOR FIRST RECOVERY OF FORMULATION – 3 (ERITEL CH- 40)
29	OPTIMIZED CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC
30	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (100 + 50 ng/ µl)
31	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (200 + 100 ng/ µl)
32	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (300 + 150 ng/ µl)
33	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (400 + 200 ng/ µl)
34	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (500 + 250 ng/ µl)

35	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (600 + 300 ng/ µl)
36	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (700 + 350 ng/ µl)
37	LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC (800 + 400 ng/ µl)
38	CALIBRATION CURVE FOR TELMISARTAN BY RP – HPTLC
39	CALIBRATION CURVE FOR CHLORTHALIDONE BY RP – HPLC
40	CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC REPEATABILITY 1
41	CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC REPEATABILITY 2
42	CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC REPEATABILITY 3
43	CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC REPEATABILITY 4
44	CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC REPEATABILITY 5
45	CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC REPEATABILITY 6
46	CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40) BY HPTLC RECOVERY- 1
47	CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40) BY HPTLC RECOVERY- 2
48	CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40) BY HPTLC RECOVERY - 3

LIST OF TABLES

TABLE NO.	CONTENT
1	SOLUBILITY PROFILE OF TELMISARTAN
2	SOLUBILITY PROFILE OF CHLORTHALIDONE
3	OPTICAL CHARACTERISTICS OF TELMISARTAN (FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)
4	OPTICAL CHARACTERISTICS OF CHLORTHALIDONE (FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)
5	ANALYSIS OF SYNTHETIC MIXTURE (FIRST ORDER DERIVATIVE SPECTROSCOPY)
6	QUANTIFICATION OF FORMULATION (ERITEL – CH 40) (FIRST ORDER DERIVATIVE SPECTROSCOPY)
7	INTRADAY AND INTER DAY ANALYSIS OF FORMULATION (FIRST ORDER DERIVATIVE SPECTROSCOPY)
8	RUGGEDNESS STUDY (FIRST ORDER DERIVATIVE SPECTROSCOPY)
9	RECOVERY ANALYSIS OF FORMULATION (FIRST ORDER DERIVATIVE SPECTROSCOPY)
10	SYSTEM SUITABILITY PARAMETERS FOR TELMISARTAN AND CHLORTHALIDONE
11	OPTICAL PARAMETER OF TELMISARTAN AND CHLORTHALIDONE BY RP-HPLC
12	QUANTIFICATION OF FORMULATION (ERITEL-CH 40) BY RP – HPLC
13	RECOVERY ANALYSIS OF FORMULATION (ERITEL-CH 40) BY RP – HPLC
14	OPTICAL CHARACTERISTICS OF TELMISARTAN AND CHLORTHALIDONE BY HPTLC
15	QUANTIFICATION OF FORMULATION (ERITEL – CH 40) BY HPTLC
16	RECOVERY ANALYSIS OF FORMULATION (ERITEL-CH 40) BY RP – HPTLC

SYMBOLS AND ABBREVIATIONS

ICH	-	International Conference on Harmonization
λ	-	Lambda
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
µg/ ml	-	Microgram Per Millilitre
mg/ tab	-	Milligram Per Tablet
ml	-	Millilitre
mM	-	Milli Mole
nm	-	Nanometer
pH	-	Negative Logarithm of Hydrogen Ion Concentration
%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
RP - HPLC	-	Reverse Phase -High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
SD	-	Standard Deviation
SE	-	Standard Error
UV-VIS	-	Ultraviolet - Visible
USP	-	United States Pharmacopoeia
IP	-	Indian Pharmacopoeia
BP	-	British Pharmacopoeia
IR	-	Infra Red
°C	-	Degree Celsius
Gms	-	Grams
µl	-	Microlitre

rpm	-	Rotations Per Minute
µ	-	Micron
v/v	-	Volume/Volume
min	-	Minute
ml/ min	-	Millilitre/minute
ng/ µl	-	Nanogram/ microlitre
hυ	-	Planck's Constant
GC	-	Gas Chromatography
USFDA	-	United States Food and Drug Administration
WHO	-	World Health Organization
GMP	-	Good Manufacturing Practice
GLP	-	Good Laboratory Practice
S/N	-	Signal to Noise ratio
m	-	Slope
c	-	Intercept
ODS	-	Octa Decyl Silane
AR	-	Analytical Reagent
TEL	-	Telmisartan
CHL	-	Chlorthalidone
NaOH	-	Sodium hydroxide
ARB	-	Angiotensin II receptor blockers
KH_2PO_4	-	Potassium dihydrgen Phosphate

INTRODUCTION

1. INTRODUCTION

1.1 INTRODUCTION TO ANALYTICAL CHEMISTRY

(*Fifeld* F.W *and Kealey D. 2006*)

Analytical chemistry is a metrological discipline that develops, optimizes and applied measurement processes intended to derive quality (bio) chemical information of global and partial type from natural and artificial objects or systems in order to solve analytical problems derived from information needs.

1.2 Aims and Objectives

Thus, Analytical chemistry has two main aims are Qualitative (intrinsic) and Quantitative (Extrinsic). The Qualitative aim is the achievement of metrological quality, i.e. ensuring full consistency between the analytical results delivered and the actual value of the measured parameters; in metrological terms, this translates into producing high traceable results subject to very little uncertainty. The Quantitative aim is solving the analytical problems derived from the (bio) chemical information needs posed by a variety of "clients".

1.3 ANALYTICAL METHOD (Douglas A. Skoog, *et al.,* 2006)

The analytical method maybe

1) Qualitative analysis
2) Quantitative analysis

Qualitative analysis was performed to establish composition of natural/synthetic substances. These tests were performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.

Quantitative analysis techniques are mainly used to quantify any compound or substance in the sample. These techniques are based in

(a) The quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained,

(b) The characteristic movement of a substance through a defined medium under controlled conditions,

(c) Electrical measurement

(d) Measurement of some spectroscopic properties of the compound.

The analytical methods are two types

- Classical methods
- Instrumental methods

In classical methods, for qualitative analysis, the analyte was extracted and treated with the reagent specific for a functional group to give a coloured reaction. In quantitative analysis, the amount of the analyte in determined by titrimetric method or by gravimetric method.

The instrumental methods are based on the physical properties of the analyte such as the light absorption or emission, conductivity, mass to charge ratio, fluorescence, adsorption and partition etc. The instrumental methods are basically categorised as follows

1.4 ANALYTICAL TECHNIQUES (*Fifeld* F.W *and Kealey D. 2006*)

A wide variety of parameters may be measured by using following techniques.

S.No	Group	Property measured
1	Gravimetric	Weight of pure analyte or of a stoichiometric compound containing it.
2	Spectrometric	Intensity of electromagnetic radiation emitted or absorbed by the analyte.
3	Electrochemical	Electrical properties of analyte solutions
4	Radiochemical	Intensity nuclear radiations emitted by the analyte.
5	Mass spectrometric	Abundance of molecular fragments derived from the analyte.
6	Chromatographic	Physio- chemical properties of individual analyte after separation.

1.5 SPECTROSCOPIC METHODS

(Gurudeep R. Chatwal, *et al*., 2008; Beckett and Stenlake, *et al*., 2007)

Spectroscopy deals with the interaction of an analyte with electromagnetic radiation. The interaction of the electromagnetic radiation results in absorption or emission radiations. Based on the absorption or emission the spectroscopy is classified into, absorption spectroscopy and emission spectroscopy.

1.5.1 Absorption Spectroscopy

When a beam of electromagnetic radiation is passed through an analyte, certain amount of the radiation is absorbed into the matter. The analyte after absorbing the radiation goes from the ground state to the excited state giving the absorption spectra. The various

absorption spectroscopies include the UV-Visible absorption, X-ray absorption, infrared absorption, microwave absorption, radio frequency absorption and atomic absorption, etc.

1.5.1.1 Atomic Absorption

In the atomic absorption spectra the electrons are excited from the lower energy state to a higher energy state by absorption of electromagnetic radiation. The atom absorbs the electromagnetic radiation of energy corresponding to the difference in energy between the higher and lower energy states of the absorbing atom. UV-Visible radiation can excite only the electrons in the outer most orbital, whereas the X-ray has the capacity to excite the electrons located in the inner shell near to the nuclei.

1.5.1.2 Molecular Absorption

The molecular absorption spectra of polyatomic molecules are more complex than the atomic absorption spectra since the number of energy states are higher. The energies associated with a molecule are rotational energy, vibrational energy and electronic energy.

$$E = E_{electronic} + E_{vibrational} + E_{rotational}$$

The molecule absorbs electromagnetic radiation of energy corresponding to the difference in the energy of the ground state molecule and the excited state molecule. The difference in energy ΔE is given by,

$$\Delta E = (E_{electronic} + E_{vibrational} + E_{rotational})_{excited} - (E_{electronic} + E_{vibrational} + E_{rotational})_{ground}$$

The UV-Visible radiations and X-rays have the energy to induce the transition from the ground state to the excited state.

1.5.2 Emission Spectroscopy

Emission spectroscopy is the technique in which the wavelength of the photons emitted by an analyte due to the transition from higher energy level to lower energy on exposure to an electromagnetic radiation was studied. Each analyte emits a specific wavelength of radiation corresponding to the composition of the sample. The energy of the photons emitted is given by

$$E_{photon} = h\upsilon$$

Where E is the energy of the photon, ν is the frequency and h is the Planck's constant. The various instrumental techniques which are based on the measurement of the emitted radiation include flame photometry, fluorimetry, radiochemical methods.

1.5.2.1. Atomic Emission

When an analyte is heated, it emits light characteristic of the atom present in it. For example, sodium when heated emits yellow light and potassium emits lilac light. When a metal is heated, the electrons in the outer orbital absorb the heat and goes to a higher energy

state. The atom then comes back to the ground state by emitting the photons of light which has energy equal to the difference between the higher energy state and the lower energy

state. The instrumental analytical technique, such as the flame emission spectroscopy works on the principle of measuring the photons of energy emitted by a thermally excited atom.

1.5.2.2. Molecular Emission

When a beam of electromagnetic radiation falls on a molecular species, it absorbs the radiation and gets excited. The excited molecules are short lived and it fall back to the ground state immediately. The molecules in the excited sate have higher vibrational energy than that of the ground state. They emit the absorbed energy by the following ways, fluorescence and phosphorescence.The difference in the energy levels of the absorbed radiation, fluorescence and phosphorescence are as below

$$\Delta E_{absorption} > \Delta E_{fluorescence} > \Delta E_{phosphorescence}$$

The analytical techniques spectrofluorimetry and phosphorimetry involves the principle of molecular emission.

1.5.3. UV Spectroscopy

UV-visible spectroscopic methods are based on the type of chromospheres/functional group present in the drug moiety. Multi component systems are also easily analysed by means of spectral isolation. Spectroscopic methods are widely used as tools for quantitative analysis, characterization and quality control in the pharmaceutical, agricultural and biomedical fields

The UV spectroscopy is one of the most widely used instrumental analytical techniques for the analysis of pharmaceuticals. The UV region extends from 190 nm to 380 nm. The instrument used to measure the intensity of the UV radiation absorbed or transmitted is known as the UV - Visible spectrophotometers. A molecule can absorb the UV radiation only when the energy of the radiation matches the energy that was required to induce electronic transition in the molecule.

1.5.3.1 Laws of Absorption

When a beam of UV light is allowed to pass through a substance which absorbs the UV light, the intensity of the transmitted light was lesser than the incident light. The reduction of the intensity is may be due to reflections on the surface of the cell, scattering of light by macro molecules and absorption.

The two important laws which govern the UV spectroscopy are the Lambert's law and Beer's law. Lambert's law states that the intensity of the light decreases exponentially with decrease in the thickness of the medium through which it passes. Beer's law states that the

intensity of the light decreases exponentially with increase in the concentration of the absorbing substance.

The two laws where combined to form the Beer-Lambert's law, which is given by the equation

$$A = abc$$

Where, A is the absorbance

a is the absorptivity

b is the path length

c is the concentration.

The absorptivity is defined as, the absorbance of a substance at a specific wavelength of 1 g/100 ml solution in a 1cm cell.

1.5.3.2 Beer - Lambert's Law

When light is incident upon a homogeneous medium, a part of incident light is reflected, a part is absorbed by the medium and the remainder is allowed to transmit as such.

$$I_0 = I_a + I_t + I_r$$

Where,

I_0 = Incident light

I_a = Absorbed light

I_t = Transmitted light

I_r = Reflected light

Lambert's Law states "when a beam of light is allowed to pass through a transparent medium, the rate of decrease of intensity with the thickness of the medium is directly proportional to the intensity of the light".

$$-\frac{dI}{dt} \propto I$$

$$I_t = I_0 e^{-kt} \quad \text{---------- (1)}$$

(or)

$$In\frac{I_0}{I_t} = kt$$

Beer's Law states "The intensity of a beam of monochromatic light decreases exponentially with the increase in concentration of the absorbing substance".

$$-\frac{dI}{dc} \propto I$$

$$I_t = I_0 e^{-k'c} \quad \text{---------- (2)}$$

(or)

$$In\frac{I_0}{I_t} = -k'c$$

By solving equations 1 and 2, on changing equations from natural logarithm,

$$I_t = I_0 . 10^{-0.4343kt} = I_0\, 10^{-kt} \quad \text{---------- (3)}$$

$$I_t = I_0 . 10^{-0.4343\, k'c} = I_0^{-k'c} \quad \text{---------- (4)}$$

On combining equations 3 and 4,

$$I_t = I_0\, 10^{-act}$$

$$\log\frac{I_0}{I_t} = act$$

Where k and k' are constants, C is the concentration of the absorbing substance and denotes thickness of the medium.

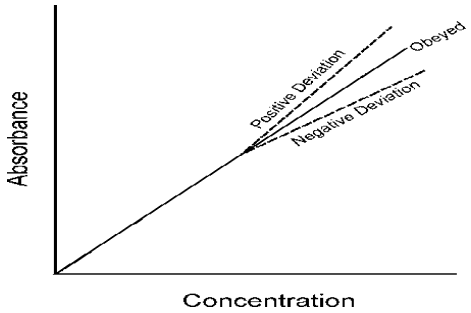
1.5.3.2.1 Limitations of Beer's law

The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Causes of nonlinearity include:

- deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity
- scattering of light due to particulates in the sample
- fluorescence or phosphorescence of the sample
- changes in refractive index at high analyte concentration
- shifts in chemical equilibria as a function of concentration
- non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band
- stray light

1.5.3.2.2 Deviations from Beer's law

According to Beer's law, a straight line passing through the origin should be obtained, when a graph is plotted between absorbance and concentration. But there is always a deviation from linear relationship between absorbance and concentration and intact the shape of an absorption curve usually changes with changes in concentration of solution and unless precautions are observed. Deviations from the law may be positive or negative according to whether the resulting curve is concave upward or concave downward.



The latter two are generally known as instrumental deviation and chemical deviation.

a. Instrumental deviations

Stray radiation, Improper slit width, Fluctuation in single beam.

b. Chemical deviations

Hydrolysis, Association, Polymerization, Ionization and Hydrogen bonding

1.5.3.2.3 Deviations from Beer's law can arise due to the following factors

1. Beer's law will hold over a wide range of concentration provided the structure of coloured ion or of the coloured non electrolyte in the dissolved state does not change with concentration. If a coloured solution is having a foreign substance whose ions do not react chemically with the coloured components, its small concentration does not affect the light absorption and may also alter the value of extinction co - efficient.
2. Deviations may also occur if the coloured solute ions dissociates or associates.
3. Deviations may also occur due to the presence of impurities that fluorescence or absorb at absorption wavelength.
4. Deviations may occur if monochromatic light is not used.
5. Deviations may occur if the width of slit is not proper and therefore it allows undesirable radiations to fall on the detector.
6. Deviations may occur if the solution undergoes polymerization.
7. Beer's law cannot apply to suspensions but the latter can estimated calorimetrically after preparing a reference curve with known concentrations.

1.5.3.3 Transitions in Organic Molecules (Gurdeep R. Chatwal, *et al*., 2008)

The absorption in the ultraviolet region results in the transition of the valence electron form the ground level to the excited level. The three types of electrons involved in the transition are

σ-electrons: These are involved in the formation of saturated bonds. The energy required for the excitation of the electrons is more than that of the UV radiations. Hence these electrons do not absorb near UV radiation.

π-electrons: These are involved in the formation of unsaturated bonds.

Example: Dienes, trienes and aromatic compounds. It absorbs radiation in near UV region.

n-electrons: These are the lone pair of electrons present in atoms such as oxygen, nitrogen etc., in a molecule. They can be excited by both UV and Visible radiations.

The various types of transitions are

σ →σ*

n→σ*

π → π *

n→π*

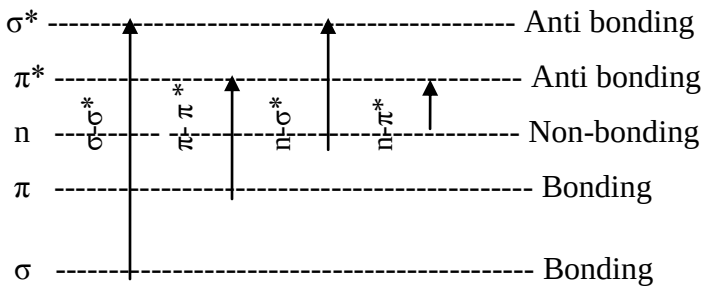


Figure: Electronic Transitions

The energy required for the various types of transitions are

σ→σ* > n→σ* > π → π * > n→ π *

1.5.3.3.1 σ→σ transitions*

These transitions occur in saturated hydrocarbons with single bonds and no lone pair of electrons. The energy required for this type of transition is very high because of the strong sigma bond formed by the valence electrons. Thus, the transitions occur at very short wavelength. The saturated hydrocarbons such as methane, ethane, propane etc. absorbs at 126 -135 nm region of the UV region. Hence these compounds are used as solvents in UV spectroscopy.

1.5.3.3.2 n → σ transitions*

Saturated compounds with lone pair of electrons show n→σ* transitions in addition to σ→σ* transitions. The energy required for the n→σ* transition is lesser than the energy required for σ→σ* transitions. The energy required for n→σ* transition, in alkyl halides, decreases with increase in the size of the halogen atom. Alcohols and amines forms hydrogen bonding with the solvent hence require higher energy for the transitions.

1.5.3.3.3 $\pi \rightarrow \pi^*$ transitions

These transitions occur in unsaturated compounds containing double or triple bonds and also in aromatic compounds. Lower energy is required for these transitions and hence a longer wavelength causes the excitation of the molecule.

1.5.3.3.4 $n \rightarrow \pi^*$ transitions

These transitions occur in compound which contains oxygen, nitrogen, sulphur and halogens because of the presence of free lone pair of electrons. These transitions require least amount of energy and hence they occur in UV and Visible region. Saturated carbonyl compounds shows two types of transitions, low energy $n \rightarrow \pi^*$ transitions occurring at longer wavelength and high energy $n \rightarrow \pi^*$ transitions occurring at lower wavelength. The shifts in the absorption of the carbonyl compounds are due to the polarity of the solvent.

1.5.3.4 Transition Probability (Gurudeep R. Chatwal, *et al*., 2008)

It is not essential that, when a compound is exposed to UV light, transition of the electron should take place. The probability that an electronic transition should take place depends on the value of extinction coefficient. The transitions are classified as allowed transition and forbidden transition.

1.5.3.4.1 Allowed Transitions

The transitions having ε_{max} value greater than 10^4 are called allowed transitions. They generally arise due to the $\pi \rightarrow \pi^*$ transitions. For example, 1, 3 – butadiene exhibits absorption maximum at 217 nm and has ε_{max} value of 21000 represents allowed transitions.

1.5.3.4.2 Forbidden Transitions

These transitions have ε_{max} value less than 10^4. They occur due to $n \rightarrow \pi^*$ transitions. Example, saturated carbonyl compound (R-C=O) shows absorption near 290 nm and ε_{max} value less than 100 represent forbidden transitions.

1.5.3.5 Chromophore

These are groups or structure which is responsible to impart colour to the compound. The presence of chromophore is responsible for the absorption of UV radiation by any compound. The groups include nitro group, amine groups, double bonds, triple bonds, etc.

There are two types of chromophore

- Groups containing π electrons and undergoes $\pi \rightarrow \pi^*$ transitions. Example: ethylene, acetylenes
- Groups containing π electrons and n electrons. They undergo two types of transition like $\pi \rightarrow \pi^*$ transitions and $n \rightarrow \pi^*$ transitions. Example:- carbonyls, nitriles, azo compounds etc.

1.5.3.6 Auxochrome

Any groups which do not itself act as a chromophore but its presence brings a shift in the position of absorption maximum. Chromophores are unsaturated whereas the auxochromes are covalently saturated. The auxochromes are of two types

Co-ordinately unsaturated- (NH2, -S- groups containing lone pair of electrons).

Co-ordinately saturated- (NH3+ groups).

1.5.3.7 Absorption and Intensity shifts

1.5.3.7.1 Bathochromic shift or Red shift

The shift in the absorption maximum of a compound, due to the presence of certain auxochromes, towards longer wavelength is called as the bathochromic shift.

1.5.3.7.2 Hypsochromic shift or Blue shift

The shift in the absorption maximum to shorter wavelength is called Hypsochromic shift. The shift is due to solvent effect or removal of conjugation in a molecule.

1.5.3.7.3 Hyperchromic effect

The increase in intensity of absorption by inclusion of an auxochrome to a system is hyperchromic shift.

1.5.3.7.4 Hypochromic shift

The decrease in the intensity of absorption is due to the distortion of the geometry of the molecule.

1.5.3.8 Solvent Effect

The solvent used for the spectral analysis should not interfere in the absorbance of the analyte. It means that the solvent should not have any absorbance in the region under investigation. Based on the polarity of the solvent used the intensity of the absorption changes for a particular analyte. The α, β – unsaturated carbonyl compounds shows two different types of transitions

$n \rightarrow \pi^*$ transition

The increase in polarity moves the absorption maximum to a shorter wavelength. The ground state is more polar when compared to the excited state.

$\pi \rightarrow \pi^*$ transitions

The increase in polarity moves the absorption maximum to longer wavelength. Only lesser energy is required for this transition and hence shows red shift.

1.5.3.9 Choice of Solvent

There are two important requirements a solvent must satisfy to be used as a solvent in UV spectroscopy.

They are

- It should be transparent throughout the region of UV under investigation
- It should not interact with the solute molecules and should be less polar.

1.5.3.9.1. Solvents used in UV spectroscopy

S.No.	Solvent	Cut-off (nm)
1.	Ethanol	205
2.	Methanol	210
3.	Acetonitrile	210
4.	Hexane	210
5.	Cyclo hexane	210
6.	Diethyl ether	220
7.	Chloroform	245
8.	Carbon tetrachloride	265
9.	Toluene	280

1.5.3.10 Instrumentation *(Gurudeep R. Chatwal et al., 2008)*

All photometers, colorimeters and spectrophotometers have the following basic components

1.5.3.10.1 *Radiation source*

i) It must be stable.

ii) It must be of sufficient intensity for the transmitted energy to be detected at

the end of the optical path.

iii) It must supply continuous radiation over the entire wavelength region in which it is used.

1.5.3.10.2 UV region

Hydrogen discharge lamp, Deuterium discharge lamp, Xenon arc Lamp

1.5.3.10.3 Visible region

The tungsten lamp and tungsten halogen lamp are the most common source of visible radiation.

1.5.3.10.4 Filters and monochromators

The filters and monochromators are used to disperse the radiation according to the wavelength.

1.5.3.10.4.1 Filters

A light filter is a device that allows light of the required wavelength to pass but absorbs light of other wavelengths wholly or partially. Thus, a suitable filter can be selecting a desired wavelength band. It means that a particular filter may be used for a specific analysis. If analysis is carried out for several species, a large number of filters have to be used and interchanged. This method is very useful for routine analysis.

Types of filters

Filters are two types,

i) Absorption filters

ii) Interference filters

Absorption filters work by selective absorption of unwanted wavelengths and are made up of solid sheet of glass, coloured by a pigment or dispersed in glass and dyed gelatin. Interference filters work by selective transmission of selected wavelengths and they are made up of semitransparent metal film deposited on a glass plate and coated with dielectric material (MgF_2).

1.5.3.10.4.2. Monochromators

Monochromators successfully isolates band of wavelengths usually much more than a narrower filter. The essential elements of a monochromator are an entrance slit, a dispersing element (prim or gratings) and an exit slit. The entrance slit sharply defines the incoming beam of heterochromatic radiation. The dispersing element disperses the heterochromatic radiation into its component wavelengths where as exit slit allows the nominal wavelength together with a band of wavelength on either side of it. The position of the dispersing element is always adjusted by rotating it to vary the nominal wavelength passing through the exit slit.

Types of monochromator

1) Prisms

2) Gratings

A prism is made up of quartz (for UV region), glass (for visual range) and alkali halides (for IR). The main advantage of prisms is that they undergo dispersion giving wavelengths which do not overlap, but the main disadvantage is that they give non – linear dispersion. A grating consists of large number of parallel lines ruled on a highly polished surface like alumina. Generally, gratings are difficult to prepare therefore, replica gratings are prepared from an original grating. This is done by coating the original grating with a film of an epoxy resin which after setting is removed to yield replica. Then replica is made reflective by aluminizing its surface. Gratings give linear dispersion but they suffer from an overlap of spectral orders.

1.5.3.10.5 Sample cells

These are containers for holding the sample and reference solutions and must be transparent to the radiation passing through generally with a thickness of 1 Cm. The choice of a sample cells are based on transmission characteristics at desired wave lengths, the path length, shape, size and the relative expense. The transmission characteristics are based on the construction materials. For UV region, the cells made up of quartz and for visible region, the cells are made of glass.

1.5.3.10.6 Detectors

Detectors used in UV-Visible spectrophotometers can be called as photometric detectors. In these detectors the light energy is converted to electrical signal which can be recorded. The types of detectors used are Barrier Layer cell (or) Photo Voltaic cell, Photo tubes (or) Photo emissive tubes, Photomultiplier tubes and Photo diode.

1.5.7.10.6. Recorders

Detectors transmits the amount of light absorbed by a particular chemical species and only by that species is desired and by correcting the absorbance of solvent and other species in the solution. The recorders record the spectrum without any interferences compared with blank and they are user friendly.

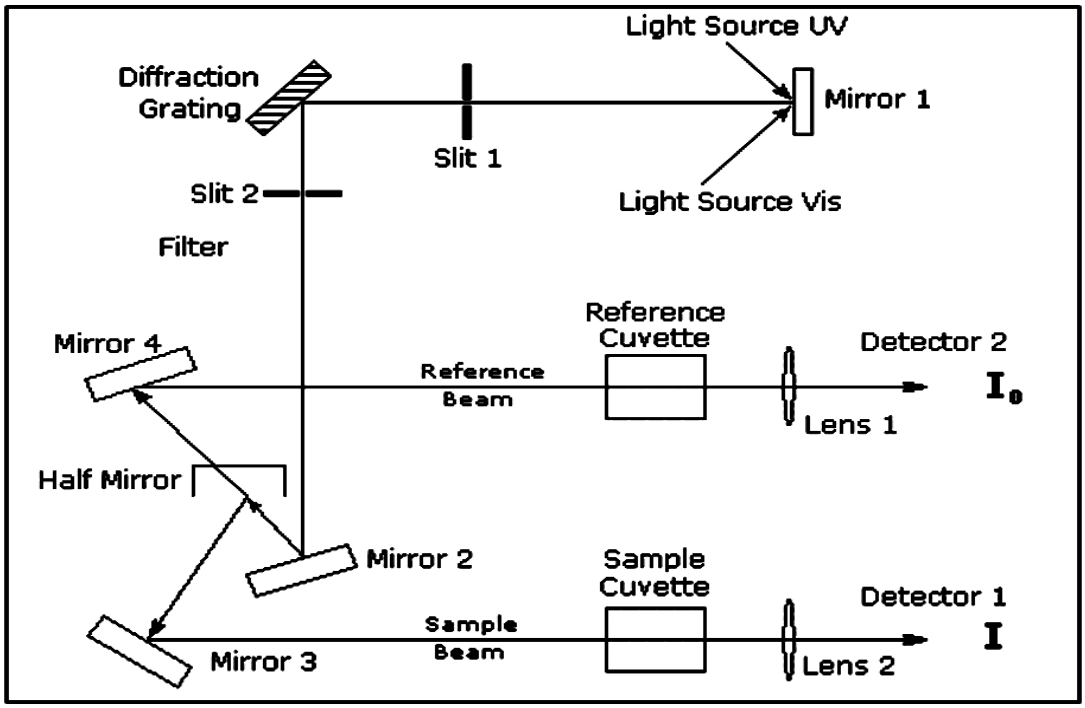


Figure: Double Beam UV - Spectrophotometer

1.5.4. Quantitative Analysis (Beckett and Stenlake, *et al*., 2007)

1.5.4.1. Quantitative Analysis of Single Component

The assay of an analyte is done by dissolving the analyte in a suitable solvent and measuring the absorbance of the solution at the required wavelength. The selected wavelength is the absorbance maximum of the analyte in that particular solvent. The concentration of the analyte can be determined by

- Use of absorptivity value
- Use of calibration graph
- Single or double point standardization

1.5.4.1.1. Absorptivity Value Method

This method is usually followed in official books such as Indian Pharmacopoeia, British Pharmacopoeia etc. The advantage of the method is, the preparation of standard solutions of reference substance is not required for the calculation of the concentration of the analyte.

1.5.4.1.2 Calibration Graph Method

In this method, a series of linear concentration solutions of the reference solutions are prepared and the value of absorbance is plotted against the concentration of the reference solution. From the graph the absorbance of the sample solution is plotted and the concentration is found.

1.5.4.1.3 Single Point or Double Point Standardization

In single point standardization, the standard and the sample solutions are prepared under same identical condition. Also, the standard and the sample concentration are almost equal. Then after the measurement of absorbance the following formula is applied to find the unknown sample concentration

$$C_{test} = \frac{A_{test}}{A_{std}} \times C_{std}$$

Double point standardization is used when there is a linear but non proportional relationship between concentration and absorbance. The concentration of one of the standard is higher and the concentration of other is lower than that of the standard.

$$C_{test} = \frac{(A_{test} - A_{std1})(C_{std1} - C_{std2}) + C_{std1}(A_{std1} - A_{std2})}{A_{std1} - A_{std2}}$$

1.5.4.2. Assay of Substance in Multi Component Samples

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of sample containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay. Alternatively, interference which is difficult to quantify may arise in the analysis of formulations from manufacturing impurities, decomposition products and formulation excipients. Unwanted absorption from these sources is termed irrelevant absorption and, if not removed, imparts a systematic error to the assay of the drug in the sample.

The basis of all the spectrophotometric techniques for multi component sample is the property that at all wavelengths;

- The absorbance of a solution is the sum of absorbance's of the individual components; or
- The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference cell.

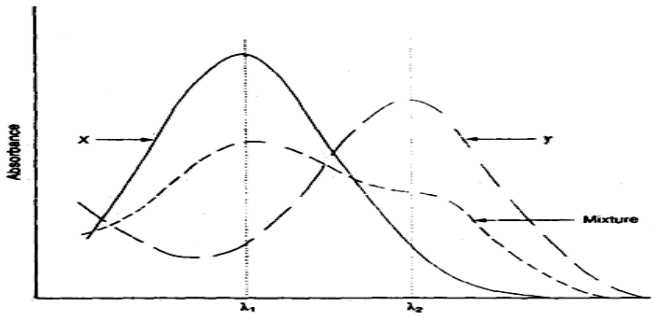
Multi component analysis is done when the sample contains more than one analyte to be quantified in the sample. In such methods one of the analyte may be taken as interferent and the absorbance of the interferent reduced to find the true absorbance of the analyte. Similarly the absorbance of the other analyte is found by taking the first analyte as the interferent. The various methods used are as follows.

- Simultaneous equation method
- Absorption ratio method

- Geometric correction method
- Orthogonal polynomial method
- Difference spectroscopy
- Area under curve method
- Absorbance ratio method
- Derivative spectrophotometry

1.5.4.2.1 Simultaneous equations method

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λmax of the other, it may be possible to determine both drugs by the technique of simultaneous equations (Vierodt's method)



The information required is:

a. The absorptivities of X at λ_1 and λ_2, a_{x1} and a_{x2} respectively.
b. The absorptivities of Y at λ_1 and λ_2, a_{y1} and a_{y2} respectively.
c. The absorbances of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.

Let Cx and Cy be the concentrations of X and Y respectively for diluted sample.

Two equations are constructed based upon the fact that at λ_1 and λ_2 the absorbance of the mixture is the sum of individual absorbances of X and Y.

At λ_1

$$A_1 = ax_1bcx + ay_1bcy \qquad (1)$$

At λ_2

$$A_1 = ax_2bc_x + ay_2bc_y \qquad (2)$$

For measurements in 1 cm cells, b = 1

Rearrange eq. (2)

$$c_y = \frac{A_2 - a_{x2}c_x}{a_{y2}}$$

Substituting for c_y in eq. (1) and rearranging gives

$$C_x = \frac{A_2 a_{y1} - A_1 a_{y2}}{a_{x2} a_{y1} - a_{x1} a_{y2}}$$

And

$$C_y = \frac{A_1 a_{x2} - A_2 a_{x1}}{a_{x2} a_{y1} - a_{x1} a_{y2}}$$

As an exercise you should derive modified equations containing a symbol (b) for path length, for application in situations where A_1 and A_2 are measured in cells other than 1 cm path length.

Criteria for obtaining maximum precision, based upon absorbance ratios, have been suggested that place limits on the relative concentrations of the components of the mixture. The criteria are that the ratios

$$\frac{A_2/A_1}{a_{x2}/a_{x1}} and \frac{a_{y2}/a_{y1}}{A_2/A_1}$$

should lie outside the range 0.1 - 2.0 for the precise determination of Y and X respectively, these criteria are satisfied only when the λ_{max} of the two components are reasonably dissimilar. An additional criterion is that the two components do not interact chemically, thereby negating the initial assumption that the total absorbance is the sum of the individual absorbance. The additivity of the absorbance should always be confirmed in the development of a new application of this technique.

1.5.4.2.2 Absorbance Ratio Method

The absorbance ratio method is a modification of the simultaneous equations procedure. It depends on the property that, for a substance which obeys Beer's Law at all wavelengths, the ratio of absorbances at any two wavelengths is a constant value independent of concentration or path length. For example, two different dilutions of the same substance give the same absorbance ratio A_1/A_2, 2.0. In the USP, this ratio is referred to as a Q value. The British Pharmacopoeia also uses a ratio of absorbance at specified wavelengths in certain confirmatory tests of identity.

In the quantitative assay of two components in admixture by the absorbance ratio method, absorbances are measured at two wavelengths one being the λ_{max} of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), i.e., an iso-absorptive point. Two equations are constructed as described

above for the method of simultaneous equation (eq. (1) and eq. (2)). Their treatment is somewhat different, however, and uses the relationship $a_x = a_{y1}$ at (λ_1).

Assume b = 1 cm.

$$A_1 = a_{x1}c_x + a_{x1}c_Y \qquad (5)$$

$$\frac{A_2}{A_1} = \frac{a_{x2}c_x + a_{y2}c_y}{a_{x1}c_x + a_{x1}c_y}$$

Divide each term by $c_x + c_y$ and let $F_x = c_x/(c_x + c_y)$ and $F_y = c_y/(c_x + c_y)$ i.e. Fx and Fy are the fractions of X and Y respectively in the mixture:

$$\frac{A_2}{A_1} = \frac{a_{x2}F_x + a_{y2}F_y}{A_{x1}F_x + a_{x1}F_y}$$

But $F_y = 1 - F_{x'}$

$$\frac{A_2}{A_1} = \frac{F_x a_{x2} - F_x a_{y2} + a_{y2}}{a_{x1}}$$

$$\frac{A_2}{A_1} = \frac{F_x a_{x2}}{a_{x1}} - \frac{F_x a_{y2}}{a_{y1}} + \frac{a_{y2}}{a_{y1}}$$

Let $Q_x = \frac{a_{x2}}{a_{x1}}, Q_y \frac{a_{y2}}{a_{y1}} and\ Q_M \frac{A_2}{A_1}$

$$Q_M = F_x(Q_x - Q_y) + Q_y$$

$$F_x = \frac{Q_M - Q_y}{Q_x - Q_y} \qquad (6)$$

Equation 6 gives the fraction, rather than the concentration of X (and consequently of Y) in the mixture in terms of absorbance ratios. As these are independent of concentration, only approximate, rather than accurate, dilutions of X, Y and the sample mixture are required to determine Q_x, Q_y and Q_M respectively.

$$A_1 = a_{x1} + (c_x + c_Y)$$

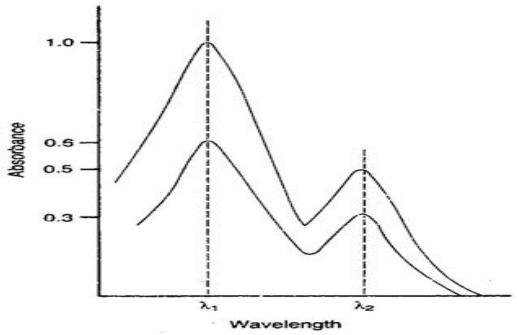
$$c_x + c_y = \frac{A_1}{a_{x1}}$$

From eq. (6)

$$\frac{c_x}{f2c_x + c_y} = \frac{Q_m - Q_y}{Q_x - Q_y b7\cdot}$$

$$\frac{c_x}{A_1/a_{x1}} = \frac{Q_M - Q_y}{Q_x - Q_y}$$

$$c_x = \frac{Q_m - Q_y}{Q_x - Q_Y} \cdot \frac{A_1}{a_{x1}} \qquad (7)$$



Equation 7, gives the concentration of X in terms of absorbance ratios, the absorbance of the mixture and the absorptivity of the compounds at the iso-absorptive wavelength. Accurate dilutions of the sample solution and of the standard solutions of X and Y are necessary for the accurate measurement of A_1 and ax_1 respectively.

1.5.4.2.3 Geometric Correction Method

A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in samples of biological origin. The simplest of these procedures is the three-point geometric procedure, which may be applied if the irrelevant absorption is linear at the three wavelengths selected.

$$\text{Corrected absorbance, D} = \frac{y(A2-A3)+ z(A2-A1)}{(Y+Z)(1-r)}$$

1.5.4.2.4 Orthogonal Polynomial Method

The technique of orthogonal polynomials is another mathematical correction procedure which involves more complex calculations than the three-point correction procedure. The basis of the method is that an absorption spectrum may be represented in terms of orthogonal functions as follows

$$A(\lambda) = p_0P_0(\lambda) + p_1P_1(\lambda) + p_2P_2(\lambda)....p_nP_n(\lambda)$$

Where, A = Absorbance

λ = Wave length

$P_o(\lambda), P_1(\lambda), P_2(\lambda)....P_n(\lambda)$ represent the polynomial coefficient

Each coefficient is proportional to each other. These polynomials represent a series of fundamental shapes and the contribution that each shape, e.g.P_2 makes to the absorption spectrum is defined by the appropriate coefficient, e.g. p_2 for P_2. The coefficients are proportional to the concentration of the absorbing analyte, and a modified Beer – Lambert equation may be constructed:

$$p_j = \alpha_j bc$$

For example, when b is 1 cm and concentration of the analyte (c), is in g/ dl. When irrelevant absorption so, present in a sample solution, the calculated coefficient (p_i) comprises the coefficients of the analyte and of the irrelevant absorption (Z).

Thus,

$$P_j = \alpha_j c + p_j(Z)$$

Where,

P_j = polynomial coefficient

a_j = proportionality constant

b = path length

c = concentration

With the correct choice of polynomial, number of wavelengths and the wavelength interval, the contribution from the irrelevant absorption may be negligible. In general, a quadratic (P_2) polynomial eliminates linear or almost linear irrelevant absorption and a cubic (P_3) polynomial eliminates parabolic irrelevant absorption.

The segment of the spectrum of the drug between $\lambda_1$and λ_8 shows a minimum around λ_3 and a maximum around λ_5. Its shape may therefore be represented by a cubic polynomial. The irrelevant absorption is a simple parabolic curve which does not contain a cubic contribution. The coefficient (P_3) of the polynomial for each set of eight absorbances (A_1……..A_8) is calculated from:

$$P_3 = [(-7) A_1 + (+5) A_2 + (+7) A_3 + (+3) A_4 + (-3) A_5 + (-7) A_6 + (-5) A_7 + (+7) A_8]$$

Where the factors are those of an eight –point cubic polynomial obtained from standard texts of numerical analysis (e.g. Fischer and Yates, 1953). The contribution of the irrelevant absorption to the coefficient of the polynomial of the sample is eliminated by the selection of these parameters, and the concentration of the drug in the sample may be calculate with

reference to a standard solution of the drug, from the proportional relationship that exists between the calculated P_3 value and concentration.

The accuracy of the orthogonal functions procedure depends on the correct choice of polynomial order and set of wavelengths. Usually, quadratic or cubic polynomials are selected depending on the shape of the absorption spectra of the drug and the irrelevant absorption. The set of wavelengths is defined by the number of wavelengths, the interval, and the mean wavelength of the set (λmax). approximately linear irrelevant absorption is normally eliminated using six to eight wavelengths, although many more, up to 20, wavelengths may be required if the irrelevant absorption contains high frequency components. The wavelength interval and λm are best obtained from convoluted absorption curve. This is a plot of the coefficient (P_j) for a specified order of polynomial, a specified number of wavelengths and a specified wavelength interval (on the ordinate) against the λm of the set of wavelengths. The optimum set of wavelengths corresponds with a maximum or minimum in the convoluted curve of the analyte and with a coefficient of zero in the convoluted curve of the irrelevant absorption. In favourable circumstances the concentration of an absorbing drug in admixture with another may be calculated if the correct choice of polynomial parameters is made, thereby eliminating the contribution of one drug from polynomial of the mixture. For, example, the selective assay phenobarbitone, combined with phenytoin in a capsule formulation using a six-point quadratic polynomial, has been reported.

The determination of the optimum set of wavelengths is readily accomplished with the aid of a microcomputer. A suitable exercise is to write a program to compute and plot the data for convoluted spectrum.

1.5.4.2.5 Difference Spectrophotometry

The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferants may be markedly improved by the technique of difference spectrophotometry. The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics.

The criteria for applying difference spectrophotometry to the assay of a substance in the presence of other absorbing substances are that:

a. reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents

b. the absorbance of the interfering substances is not altered by the reagents.

The simplest and most commonly employed technique for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acid, alkali or buffers. The ultraviolet-visible absorption spectra of many substances containing ionisable functional groups, e.g. phenols, aromatic carboxylic acids and amines, are dependent on the state of ionization of the functional groups and consequently on the pH of the solution.

The absorption spectra of equimolar solutions of Phenylephrine, a phenolic sympathomimetic agent, in both 0.1M hydrochloric acid (pH 1) and 0.1M sodium hydroxide (pH 13) are shown in figure. The ionization of the phenolic group in alkaline solution generates an additional n (non-bonded) electron that interacts with the with the ring π electrons to produce a bathochromic shift of the λ_{max} from 271nm in acidic solution to 291 nm and an increase in absorbance at the λ_{max} (hyperchromic effect). The difference absorption spectrum is a plot of the difference in absorbance between the solution at pH 13 and that at pH 1 against wavelength. It may be generated automatically using a double-beam recording spectrophotometer with the solution at pH 13 in the sample cell and the solution at pH 1 in the reference cell. At 257 and 278 nm both solutions have identical absorbance and consequently exhibit zero difference absorbance. Such wavelengths of equal absorptivity of the two species are called isobestic or iso-absorptive points. Above 278 nm the alkaline solution absorbs more intensely than the acidic solution and the ΔA is therefore positive. Between 257 and 278 nm it has a negative value. The measure value in a quantitative difference spectrophotometric assay is the ΔA at any suitable wavelength measured to the baseline, e.g. ΔA_1 at λ_1 or amplitude between an adjacent maximum and minimum, e.g. ΔA_1 at λ_2 and λ_1.

$$\text{At } \lambda_1 \ \Delta A = A_{alk} - A_{acid}$$

Where A_{alk} and A_{acid} are the individual absorbances in 0.1M sodium hydroxide and 0.1M hydrochloric acid solution respectively. If the individual absorbance, and are proportional to the concentration of the analyte and path length, the also obeys the Beer – Lambert's law and a modified equation may be derived. Where ΔA is the difference absorptivity of the substance at the wavelength of measurement.

If one or more other absorbing substances are present in the sample which at the analytical wavelength has identical absorbance in the alkaline and acidic solutions, its interference in the spectrophotometric measurement is eliminated. The selectivity of the ΔA procedure depends on the correct choice of the pH values to induce the spectral change of the

analyte without altering the absorbance of the interfering components of the sample. The use of 0.1M sodium hydrochloric acid to induce the ΔA of the analyte is convenient and satisfactory when the irrelevant absorption arises from pH intensive substances.

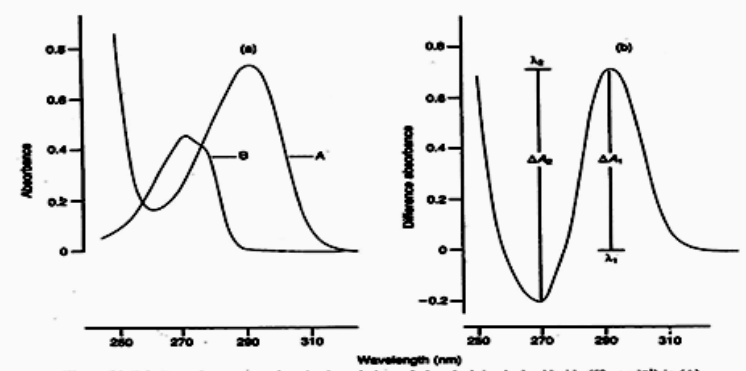


Fig. (a) The absorption spectra of equimolar solutions of phenylephrine hydrochloride (50 μg ml⁻¹) in (A) 0.1M sodium hydroxide and (B) 0.1M hydrochloric acid. (b) The difference absorption spectrum of solution B relative to solution A.

1.5.4.2.6 Area under the curve method

From the spectra obtained for calculating the simultaneous equation, the area under the curve were selected at a particular wavelength range for both the drugs were each drug have its absorption. The "X" values of the drugs were determined at the selected AUC range. The "X" value is the ratio of area under the curve at the selected wavelength range with the concentration of the component in mg/ml. These "X" values were the mean of six independent determinations. A set of two simultaneous equations were obtained by using mean "X" values. And further calculations are carried out to obtain the concentration of each drug present in the sample.

1.5.4.2.7 Absorbance correction method

The method can be used to calculate the concentration of component of interest found in a mixture containing it along some unwanted interfering component. The absorption different between two points on the mixture spectra is directly proportional to the concentration of the component to be determined irrespective of the interfering component. If the identity, concentration and absorptivity of the absorbing interferences are known, it is possible to calculate their contribution to the total absorbance of a mixture. The concentration of the absorbing component of interest is then calculated from the corrected absorbance (total absorbance minus the absorbance of the interfering substance) in a usual way. The data required for the construction of absorbance corrected for interference are

i. The λ_{max} of the drugs should be found out by using reference standards of the drugs.

ii. The calibration curve is plotted for each drug and linearity range should be found out.

iii. At one wavelength, one of the drugs shows no absorbance. Hence the other drug was calculated without any interference.

iv. The absorbance values of every drug at the two wave lengths should be measured and the absorptivity values should be calculated.

v. In another wavelength, the absorbance corrected for another drug and the first drug was determined.

1.5.4.2.8 Derivative spectrophotometry

Derivative spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, Zeroth order or D° spectrum. The first derivative (D^1) spectrum is a plot of the ratio of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum against wavelength or a plot of $dA/d\lambda$ Vs λ. At λ_2 and λ_4, the maximum positive and maximum negative slope respectively in the D°. Spectrums correspond with maximum and a minimum respectively in the D^1 spectrum. The λ_{max} at λ_3 is a wavelength of zero slope and gives $dA/d\lambda = 0$, i.e. a cross-over point, in the D^1Spectrum.

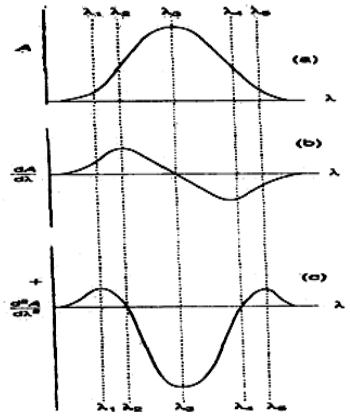


Figure: The zeroth (a), first (b) and second (c) derivative spectra of a Gaussian band

The second derivative (D^2) spectrum is a plot of the curvature of the D° spectrum against wavelength or a plot of $d^2A/d\lambda^2$ Vs λ. The maximum negative curvature at λ_3 in the D° spectrum gives a minimum in the D^2 spectrum, and at λ_1 and λ_5 the maximum positive curvature in the D° spectrum gives two small maxima called 'satellite' bands in the D^2

spectrum. At λ_2 and λ_4 the wavelengths of maximum slope and zero curvature in the D° spectrum correspond with cross-over points in the D^2 spectrum.

In summary, the first derivative spectrum of an absorption band is characterized by a maximum, a minimum, and a cross-over point at the λ_{max} of the absorption band. The-second derivative spectrum is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band. As an exercise, you should construct third and fourth derivative spectra (i.e. plots of $d^3A/d\lambda^3$ and $d^4A/d\lambda^4$ respectively against wavelength) of the fundamental spectrum.

These spectral transformations confer two principal advantages on derivative spectrophotometry. Firstly, an even order spectrum is of narrower spectral bandwidth than its fundamental spectrum. A derivative spectrum therefore shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands. Secondly, derivative spectrophotometry discriminates in favors of substances of narrow spectral bandwidth against broad bandwidth substances. This is because 'the derivative amplitude (D), i.e. the distance from a maximum to a minimum, is inversely proportional to the fundamental spectral bandwidth (14') raised to the power (n) of the derivative order.

Thus, $$D \propto (1/W)^n$$

Consequently, substances of narrow spectral bandwidth display larger derivative amplitudes than those of broad bandwidth substances.

(a) The individual spectra of two components X and Y in admixture and their combined spectrum (b) The second derivative spectrum of the mixture showing improved resolution of the individual bands.

These advantages of derivative spectrophotometry, enhanced resolution and bandwidth discrimination, permit the selective determination of certain absorbing substances in samples in which non-specific interference may prohibit the application of simple Spectrophotometric methods. For example, benzenoid drugs such as Ephedrine Hydrochloride, displaying fine structure of narrow spectral bandwidth in the region 240 - 270 nm, are both weakly absorbing (A about 15) and formulated at a relatively low dose in solid dosage preparations (typically 1 - 50 mg/ unit dose). The high excepients/drug ratio and high sample weight required for the assay may introduce into simple Spectrophotometric procedures serious irrelevant absorption from the formulation excepients. Second derivative spectrophotometry discriminates in favour of the narrow bands of the fine structure of the benzenoid drugs and eliminates the broad band absorption of the excepients. All the

amplitudes in the derivative spectrum are proportional to the concentration of the analyte, provided that Beer's Law is obeyed by the fundamental spectrum. The measured value in a quantitative assay is the largest amplitude that is unaffected by the presence of other, absorbing components of the sample spectrophotometric methods. The enhanced resolution and bandwidth discrimination, increases with increasing derivative order. However, it is also found that the concomitant increase in electronic noise. Inherent in the generation of the higher order spectra, the consequent reduction of the signal-to-noise ratio, place serious practical limitations on the higher order spectra.

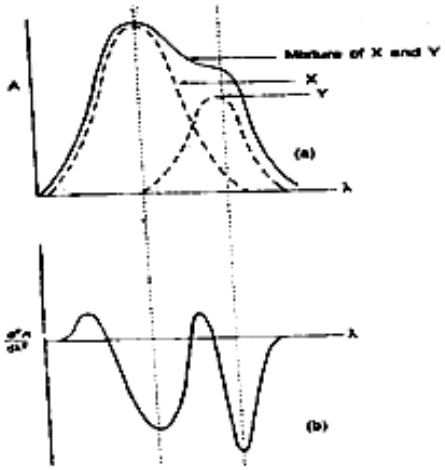


Figure: (a) The individual spectra of two components X and Y in admixture and their combined spectrum (b) The second derivative spectrum of the mixture showing improved resolution of the individual bands.

1.6. CHROMATOGRAPHY

1.6.1. INTRODUCTION TO CHROMATOGRAPHY (*Gurudeep R. Chatwal et al, 2008)*

Chromatography (Greek words Chroma - colour, Graphos - writing)

Chromatography is a non- destructive procedure for resolving a multi component mixture of trace, minor, major constituents into its individual fractions. Different variation may be applied to solid, liquids, and gases. While chromatography may be applied both quantitatively, it is a primarily a separation tool. Chromatography is relative new techniques which was first invented by M.Tswett a botanist in 1906 in warsaw. Advances have since been made and the method is used to separate colored as well as colourless substances. Chromatography may be regarded as a method of separation of solutes occurs between a stationary phase and mobile phase. Chromatography is probably the most important signal

analytical techniques used today and will probably continue to be so far the foreseeable future.

1.6.2. Definition of Chromatography

Chromatography maybe defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases.

Essentially, the technique of chromatography of separation in general involves the rate at which the components of a mixture move through a porous medium (called mobile phase) under the influence of some solvent or gas (called moving phase).

The chromatography method of separation of separation in general, involves the following steps

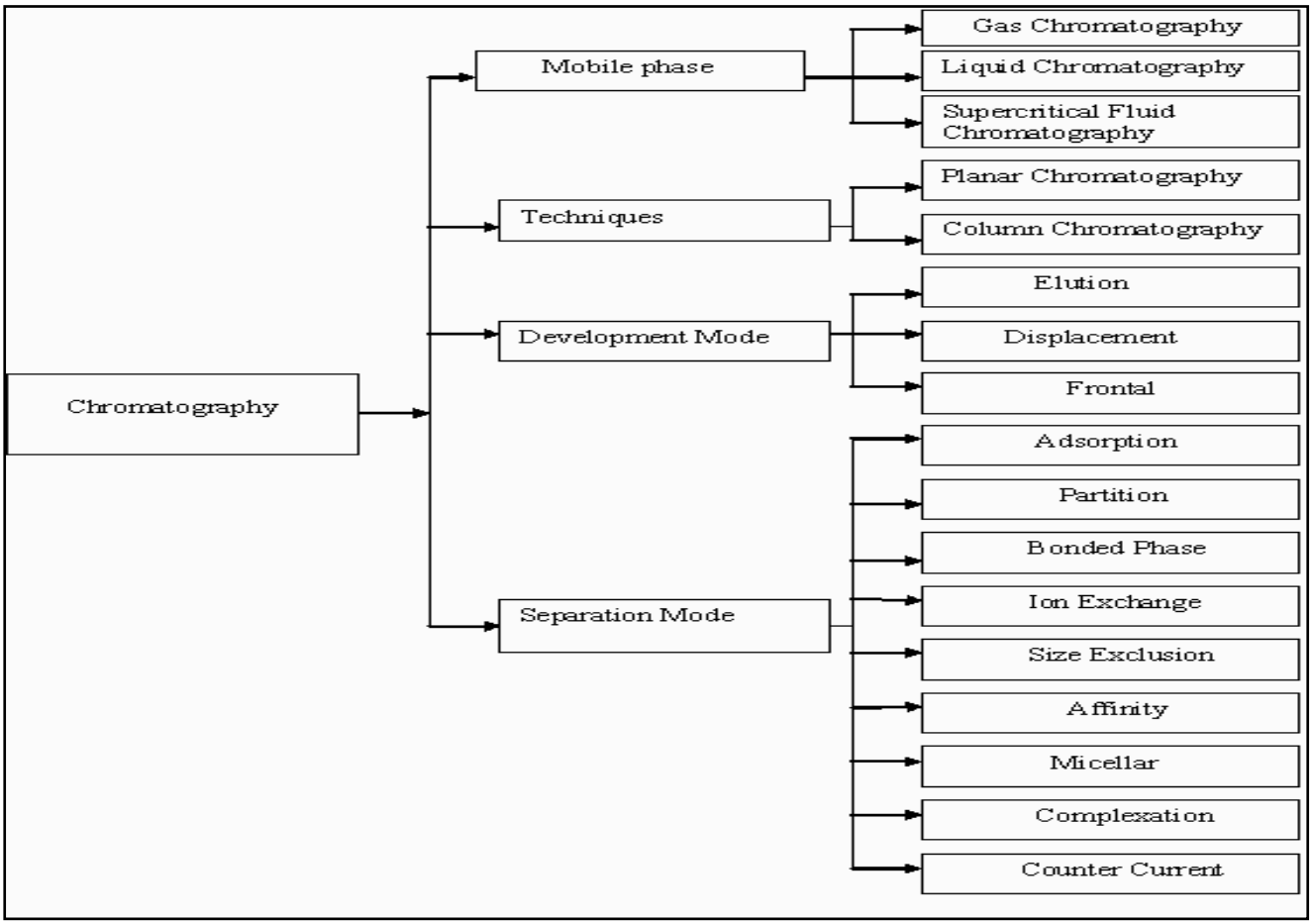
1. Adsorption or retention of a substance or substances on the stationary phase

2. Separation of adsorbed substances by the mobile phase

3. Recovery of the separate substances by a continuous flow of the mobile phase; the method being called elution.

4. Qualitative and quantitative analysis of the eluted substances.

1.6.3.TYPES OF CHROMATOGRAPHY



Chromatography techniques are roughly classified on the basis of purpose for which they are used and method developed. The different types of laboratory techniques used in the separation of mixtures are grouped under an umbrella term, chromatography. The process through which constituents of a mixture are separated and analyzed by physical means is referred to as chromatography. Apart from the different criteria of classification of chromatography discussed below, the basic criterion is the purpose for which this process is carried out. On the basis of this criterion, the process of chromatography is classified into analytical and preparative. The former is carried out for the purpose of measuring the amount of an analyte present in a mixture. On the other hand, preparative chromatography is used for separating the components of a mixture for their further use. Depending on the techniques used in chromatography, the process is broadly classified as adsorption and partition chromatography. An attempt to explain the different types of chromatography is made through this article. Let us find more about the different procedures.

1.6.3.1. Adsorption Chromatography

In this form of chromatography, the chemical mixtures in question are passed over an adsorbent bed. Different compounds present in the mixture get adsorbed on the bed at different rates. This process is mostly carried out for analytical separation. Adsorption chromatography is further divided into 'affinity' and 'ion-exchange' chromatography.

1.6.3.2. Ion-exchange Chromatography

The mechanism of ion-exchange which is used in this form of chromatography allows carrying out the segregation of analytes. This kind of segregation/separation can be performed in 2 different modes, i.e. planar and column. Separation of charged compounds like peptides, amino acids, proteins, etc. takes place through a charged stationary phase.

1.6.3.3.Column Chromatography

The column chromatography technique uses a set-up in which the stationary phase is placed in a column. There are two ways through which the stationary phase is positioned in a column: either it entirely fills the column or lines the walls of the column.

1.6.3.4. Planar Chromatography

The stationary phase is placed on a plane surface. The set-up is unlike the one used in column chromatography where stationary phase is placed in a column. Here, a plane surface is used. The plane surface could be anything from paper to glass.

1.6.3.5. Affinity Chromatography

The non-covalent interaction which takes place between the analyte in question and certain molecules is the basis of working of affinity chromatography. Purification of proteins bound to tags is conducted with this technique.

1.6.3.6. Partition Chromatography

In this separation technique, components of the given mixture are separated through the use of partition of a solute between two solvents. In the process, one of the solvents is immobilized by means of a substance present in the filter paper or column.

1.6.3.7. Gel Filtration Chromatography

This technique is also known as gel permeation or size exclusion chromatography. Molecules of the mixture in question are separated on the basis of their size. Technically speaking, the process of separation is carried out on the basis of hydrodynamic diameter

(size) of molecules. Larger molecules of the mixture are unable to enter the pores of media; therefore, molecules are washed out quickly. On the other hand, smaller molecules take more time to elute because they are able to enter the pores of media.

1.6.3.8. High Performance Liquid Chromatography

In this type of chromatography, separation of compounds is carried out on the basis of their idiosyncratic polarities. Interaction of these compounds with the stationary phase of the column too is considered. Equipment needed for carrying out high performance liquid chromatography includes a pump (used for moving the mobile phase and analyte through the column), stationary phase and a detector. Retention time for the analyte is also provided by the detector. Depending on the strength of interactions taking place between the analyte and stationary phase, retention time can vary.

1.6.3.9. Gas Chromatography

This form of chromatography uses cylinders wherein gas is stored under pressure. These gases do the work of carrying the solute. The carrier gas that is commonly used in this chromatography is helium. Flame ionization detectors and thermal conductivity are used in gas chromatography. There are three sub-types of gas chromatography which include the following: gas-liquid chromatography, gas adsorption chromatography and capillary gas chromatography. In gas-liquid chromatography, an inert porous solid is used as the stationary phase. The stationary phase used in gas chromatography is a bed formed by an adsorbent. In capillary gas chromatography, the adsorbents form a layer on fused silica or glass which line the capillary walls.

1.6.3.10. Pyrolysis Gas Chromatography

This method of chromatography makes use of pyrolysis i.e. decomposition of the sample with the help of thermal power. The process of pyrolysis is followed by the regular procedure of gas chromatography. Resistive heating, inductive heating and heating in isothermal furnace are the three methods used for carrying out pyrolysis in this technique. The volatile fragments formed by heating (at a temperature of 600-1000 °C) are separated by means of gas chromatography.

1.6.3.11. Reverse-phase Chromatography

This technique employs a method which is just opposite to that of normal phase chromatography. In reverse-phase chromatography, the stationary phase is made up of

hydrophobic compounds; they attract the hydrophobic compounds present in the mobile phase. Here, the polarity of mobile phase is reduced in order to allow the hydrophobic molecule to elute. The technique of chromatography which is meant for separation of compounds from mixtures thus, holds immense importance in fields like biochemistry, biotechnology and many others. An attempt to list as many types of chromatography as possible is made in this write-up.

1.6.4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

(Sharma B.K., 2006)

The high performance liquid chromatography is thus a method of separation in which the stationary phase is contained in a column, one end which is attached to a source of pressurized liquid eluent (mobile phase).

The choice of mobile phase is very important in HPLC and the eluting power of the mobile phase is determined by it's over all polarity of the stationary phase and the nature of sample components. For normal phase separations the eluting power increases with increasing polarity of the solvent, but for reversed phase separations, eluting power decreases with increasing solvent polarity.

1.6.4.1. Elution techniques *(Sharma B.K., 2006)*

Two types of elution techniques generally used. They are,

i) Isocratic elution

One particle solvent or mixture is pumped through the whole analysis.

ii) Gradient elution

For some determinations the solvent composition may be altered gradually gradient elution system can be classified as low pressure and high pressure system. In low pressure gradient elution system the eluent components are miner proportion varying with time at low pressure and the mixture is pumped in order to be delivered at high pressure to the column. In high pressure gradient elution system components or mixtures of fixed composition are each pumped by separate pump and then mixed at high pressure in a ratio varying with time.

1.6.4.2. HPLC SEPARATION MODES

Chromatographic Mode	Abrv.	Column Type	Mobile Phase	Application
Reverse-phase chromatography	RPC	Non-polar (e.g. C_{18})	Polar mixture of water and organic solvent (e.g. acetonitrile)	Water-soluble samples
Normal-phase chromatography	NPC	Polar (e.g. unbonded silica)	Less-polar (than stationary phase) mixture of organic solvents (e.g. hexane, ethyl ether, chloroform, methylene chloride)	Water-insoluble samples, isomer separation, and preparative HPLC
Non-aqueous reverse-phase chromatography	NARP	Non-polar (e.g. C_{18})	Mixture of organic solvents (e.g. ACN + methylene chloride)	Very hydrophobic samples
Hydrophilic interaction chromatography	HILIC	Polar (e.g. silica or amide-bonded)	Mixture of water organic solvents (e.g. ACN + H_2O	Highly polar samples that are poorly retained by reverse-phase mode
Ion-exchange chromatography	IEC	Usually an organic resin that has charged groups able to bind ions of opposite charge	Aqueous solution of a salt with buffer.	Separating ionizable samples, large biomolecules (e.g. proteins, carbohydrates)

Chromatographic Mode	Abrv.	Column Type	Mobile Phase	Application
Ion-pair chromatography	IPC	Non-polar (e.g. C_{18})	Polar mixture of water and organic solvent (e.g. ACN) w/ an ion-pairing reagent (e.g. alkylsulfonates, trifluoroacetic acid) that interacts w/ sample ions of oposite charge	Acids and bases that are weekly retained by reverse-phase
Size-exclusion chromatography	SEC	Inert	Aqueous or organic	Large biomolecules, polymers. Separates by molecular weight.

*(*http://www.justchromatography.com/chromatography/hplc-separation-modes)

1.6.4.3. HPLC stationary phases *(Robert D. Braun, 1986)*

HPLC can be performed by using the stationary phases. Generally the stationary phases are packed into a stainless steel column of 10-, 15-, 25-, 50-, 100-cm length with a diameter which is usually between 2 and 6 mm for analytical columns. Alumina is solid adsorbent which is widely used as a column packing material because alumina is basic it retains acidic compounds. Silica gel is also used as a packing material in the form of pure particles and as a pellicle on a solid support. A pellicle is a thin layer or coating on a surface generally the pellicles used for HPLC have thickness about 1µm on glass beds which have diameter about 40mm.

1.6.4.4. Mobile phases

In HPLC, for solid adsorbent and liquid stationary phases (both normal and reverse phase) mixed solvents are used as the mobile phase .Generally the mobile phase consists of a mixture of polar solvents such as alcohol and non-polar solvents such as hydro carbons.

The mobile phase must be chosen so as not to interfere with the measurement by the detector. For example, if an UV absorption detector is used the solvent cannot absorb UV radiation. Mixtures of methanol, ethanol or propanol with heptane and of chloroform with heptane are popular choices of HPLC mobile phase.

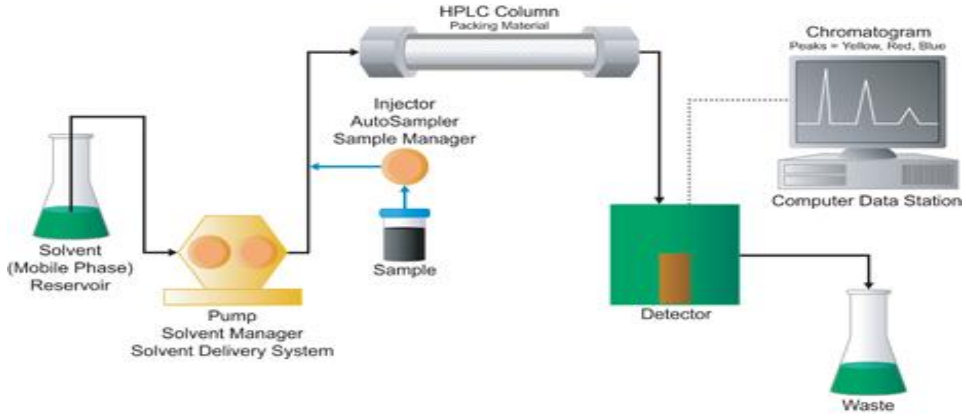
1.6.5. INSTRUMENTATION *(Willard et al., 1986)*

The general instrumentation for HPLC incorporates the following components:

i. Mobile phase reservoir pump
ii. Injector
iii. Column
iv. Detector and Data system

1.6.5.1. Mobile phase reservoir pump

The mobile phase must be delivered to the column over a wide range of flow rates and pressures to permit the use a wide variety of organic and inorganic solvents. The pump it seals and all connections must be made of materials chemically resistant to the mobile phase. A degasser is needed to remove the dissolved air and other gases from the solvent. Another desirable feature is the solvent delivery system is the capability for generating a solvent gradient. In HPLC pump should be able to operate to at least 100atm (1500 psi). A pressure suited to less expensive chromatographs however, 400atm (6000psi) is a more desirable pressure limit.



HPLC basic instrumentation *(*www.chromotech.com/ab/in/tech*)*

Standard HPLC pump requirements are,

a. Flow rate range 0.01 to 100 ml/ min.
b. Pressure range: 1 to 5000 psi.
c. Flow rate stability not more than 1%
d. Pressure pulsations less than 1%

The types of pumps used are,

1. Reciprocating Piston Pumps
2. Syringe-Type Pumps
3. Constant-Pressure Pumps

The common problems in pumping liquids are,

a. Solvent degassing
b. Corrosion
c. Compressibility

1.6.5.2. Injectors

In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference and loss in efficiency or all of these. It is always best to remove particles from the sample by filtering or centrifuging since continuous injections of particulate material will eventually cause blockage of injection devices or columns. Standard HPLC injector should be accurate in injecting volume in the range of 0.1 to 100 ml with high reproducibility and under high pressure (up to the 4000 psi) and should produce minimum band broadening and should minimize possible flow disturbances.

1.6.5.3. HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of

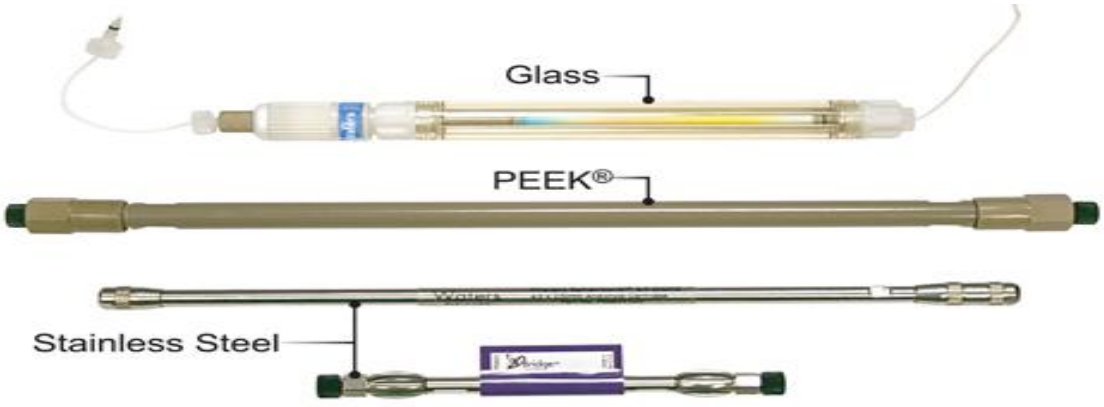
real samples as opposed to the common test samples. LC columns, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular, very expensive optically pure compounds can be used to make chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

Different types of columns are used. They are,

1. Analytical column
2. Short column
3. Narrow bore column
4. Guard column
5. Inline filters

Analytical column variables are as follows:

a. Length (10-30 cm)
b. ID (4-10 mm)
c. Packing (many kinds)
d. Particles sizes (3-10 μm)
e. Most common columns 250mmx4.6mm i.e. with 5μm particle size.



(http://www.waters.com/webassets/cms/category/media/other_images/primer_M-1_Columnexamples.jpg)

1.6.5.4. Detectors

Optical detectors are most frequently used. Current LC detectors have wide dynamic range normally allowing both analytical and preparative scale runs on the same instrument.

Basic detector requirements

An ideal LC detector should have the following properties:

1. Low drift and noise level (particularly crucial in trace analysis).
2. High sensitivity.
3. Fast response.
4. Wide linear dynamic range (this simplifies quantification).
5. Low dead volume (minimal peak broadening).
6. Cell design which eliminates remixing of the separated bands.
7. Insensitivity to changes in type of solvent, flow rate, and temperature.
8. Operational simplicity and reliability.
9. It should be tunable so that detection can be optimized for different compounds.

1.6.5.4.1. On-line detectors

A. Refractive index.
B. UV/Visible Fixed wavelength.
C. UV/Visible Variable wavelength.
D. UV/Visible Diode array.
E. Fluorescence.
F. Conductivity.
G. Mass-spectrometric (LC/MS).

1.6.5.4.2. Off-line detector

A. FTIR spiral disk monitor.

1.6.5.6. Noise and drift

The problem is to distinguish between the actual component and artifact caused by the pressure fluctuation, bubble, compositional fluctuation, etc. If the peaks are fairly large, one has no problem in distinguishing them. However, the smaller the peak, the more important is that the baseline be smooth, free of noise and drift. Baseline noise is the short time variation of the baseline from a straight line. Noise is normally measured "peak-to-peak": i.e., the distance from the top of one such small peak to the bottom of the next. Noise is the factor

which limits detector sensitivity. In trace analysis, the operator must be able to distinguish between noise spikes and component peaks. The baseline should deviate as little as possible from a horizontal line. It is usually measured for a specified time, e.g., 1/2 hour or one hour and called drift. Drift usually associated to the detector heat-up in the first hour after power-on.

1.6.5.7. Data systems

The main goal in using electronic data systems is to increase analysis accuracy and precision while reducing operator attention. In routine analysis, where no automation is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

The advantages of intelligent processors in chromatographs are,

- Additional automation options become easier to implement.
- Complex data analysis becomes more feasible.
- Software safeguards can be designed to reduce accidental misuse of the system.

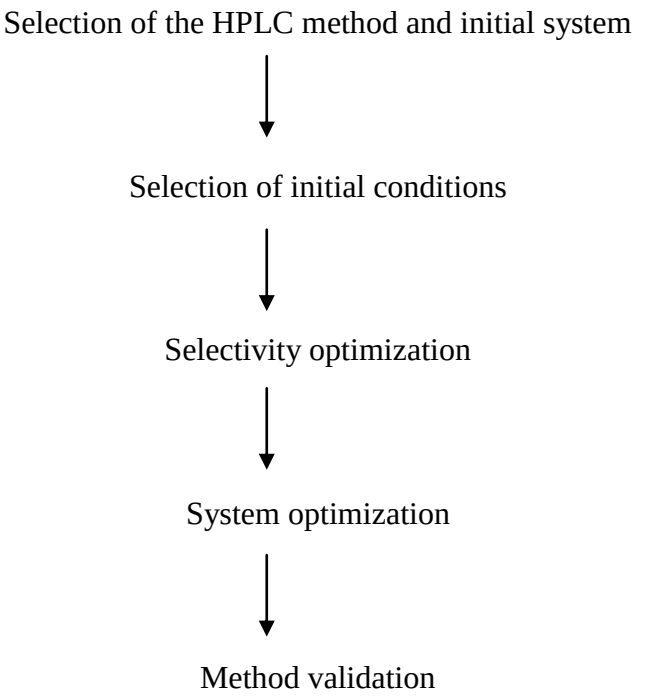
1.6.5.8. Summary of HPLC operation

1. Filter and degas mobile phase.
2. Prime pump, rinse column with strong solvents and equilibrate column.
3. Purge injection and make sure there are no air bubbles in the sample syringe.
4. Perform system suitability test.
5. Analyze sample.
6. Process and report data.
7. Rinse column and shut down pump and lamps.

1.6.7. HPLC method development

The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex. The process is influenced by the nature of the analytes. The different stages in method development was given in the following flow chart

HPLC Method development flow chart



1.6.6 Checking for problems *(Lloyd et al., 1997)*

As method development proceeds, various problems can arise, some of which are listed below.

Prolems	**Comments**
Low plate numbers	Poor choice of column, poor peak shape effect.
Column variability	Poor choice of column, secondary retention effect.
Short column life	Poor choice of column, need for sample pretreatment.
Retention drift	Insufficient column equilibration, need for sample pre-treatment, loss of bonded phase.
Poor quantitative precision	Need for better calibration.

1.6.8. QUANTITATIVE ANALYSIS

The four primary techniques for quantitation are

a. Normalized peak area

b. External standard calibration method

c. Internal standard calibration method

d. method of standard addition

1.6.8.1. Normalized peak area

After completion of a run and the integration of all significant peaks in the chromatogram, the total peak area can then be calculated. The area percent of any individual peak is referred to as the normalized peak area. The technique of normalized peak area is actually not a calibration method per se, since there is no comparison to a known amount for any peak in the chromatogram. However, this technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material.

1.6.8.2. External standard calibration method

The most general method for determining the concentration of an unknown sample is to construct a calibration plot using external standards. Standards solutions are prepared at known concentrations. A fixed volume of each standard solution is injected and analyzed, and the peak responses are plotted Vs concentration. The standard solutions are referred to as external standards, since they are prepared and analyzed in separate chromatograms from those of the unknown samples. Unknown samples are then prepared, injected and analyzed exactly in the same manner.

1.6.8.3. Internal standard calibration method

Another technique for calibration involves the addition of an internal standard to the calibration solutions and samples. The internal standard is a different compound form the analyte, but one that is well resolved in the separation. The internal standard can compensate for changes in sample size or concentration to instrumental variations. With the internal standard method, a calibration plot is produced by preparing and analyzing calibration solutions containing different concentration of the compound of interest with a fixed concentration of the internal added.

The Internal standard comply the following requirements:

i. Well resolved from the compound of interest and other peaks.
ii. Similar retention (k) to the analyte.
iii. Should not be in the original sample.
iv. Should mimic the analyte in any sample preparation steps.
v. Does not have to be chemically similar to analyte.
vi. Commercially available in high purity.
vii. Stable and unreactive with sample or mobile phase.

viii. Should have similar detector response to the analyte for the concentration used

ix. It must be separated from all compounds of interest in the separation.

1.6.8.4. Method of standard addition

A calibration standard ideally should be prepared in a blank matrix of drug formulation components without the drug substance or an animal without added compound usually can be used for standard calibration solutions. The method of standard addition is most often used in trace analysis. In this approach, different weights of analyte(s) are added to the sample matrix, which initially contains an unknown concentration of the analyte. Extrapolation of a plot of response found for the standard addition calibration concentration to zero concentration defines the original concentration in the unspiked sample.

1.6.9. System Suitability Parameters (Lloyd, 1997; Beckett and Stenlake, 2007)

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

The parameters that are affected by the changes in chromatographic conditions are,

- Column capacity factor (K_A)
- Resolution (R_s)
- Selectivity (α)
- Column efficiency (N) and
- Peak asymmetry factor (A_s)
- Tailing factor (T)

1.6.9.1. Column capacity factor (K_A)

The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of these are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (K), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

$$K_A = \frac{V_A - V_0}{V_0} = \frac{t_A - t_0}{t_0}$$

Where,

V_A = Elution volume of A

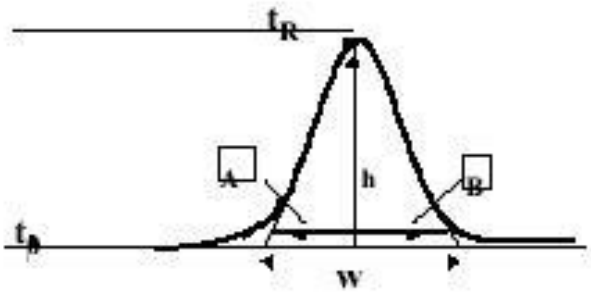
V_0 = Elution volume of a non-retained compound (void volume)

At constant flow rate, retention times (t_A and t_o) can be used instead of retention volumes. Retention data is sometimes expressed, relative to a known internal standard (B). The ratio of retention times (t_A/t_B) can be used, but the ratio of adjusted retention times $\left(\frac{t_A - t_o}{t_B - t_o}\right)$ is better when data need to be transferred between different chromatographs.

The values of 'k' of individual bands are increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \qquad k' = \phi \frac{C_s}{C_m}$$



1.6.9.2. Resolution (R_s)

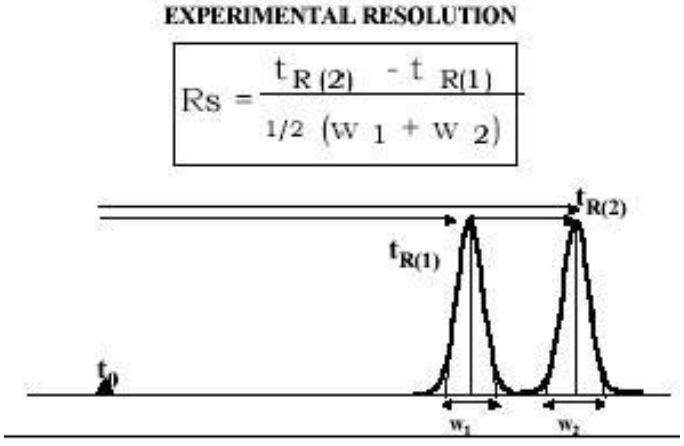
The resolution, R_s of two neighbouring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 2.0. It is calculated by using the formula,

$$R_f = \frac{Rt_2 - Rt_1}{0.5\,(W_1 + W_2)}$$

Where,

Rt_1 and Rt_2 are the retention times of components 1 and 2

W_1 and W_2 are peak widths of components 1 and 2.

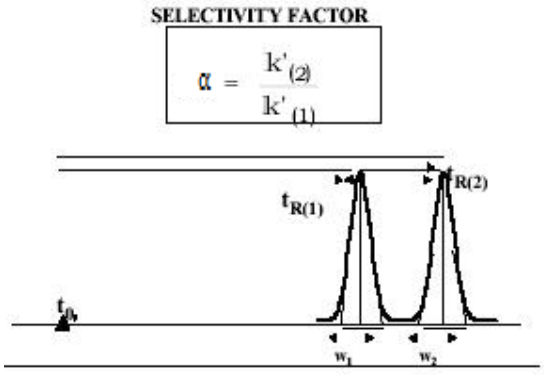


1.6.9.3. Selectivity (α)

The selectivity (or separation factor), α, is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak, respectively.



1.6.9.4. Column efficiency

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\,\frac{Rt^2}{W^2}$$

Where,

Rt is the retention time and W is the peak width.

1.6.9.5. Peak asymmetry factor (A_s)

Peak asymmetry factor, $A_s$can be used as a criterion of column performance. The peak half width b of a peak at 10 % of the peak height, divided by the corresponding front half width a gives the asymmetry factor.

$$A_S = \frac{b}{a}$$

1.6.9.6. Tailing factor (T)

The tailing factor T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision becomes less reliable.

$$\mathrm{T} = \frac{\mathrm{W}_{0.09}}{2\mathrm{f}}$$

Where,

$W_{0.05}$ = width of peak at 5% height

f = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline. Limit: ≤ 2 is preferable.

1.6.9.7. Height Equivalent to a Theoretical Plate (HETP)

A theoretical plate can be of any height, which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more, the column is less efficient. The height equivalent to a theoretical plate (HETP) is given by

$$\mathrm{HETP} = \frac{\text{Length of the column}}{\text{No. of the theoretical plates}}$$

1.6.10. High Performance Thin Layer Chromatography (Sethi, *et al*., 1996)

High Performance Thin Layer Chromatography is a versatile separation technique and is official in most of the Pharmacopoeias for determining content uniformity, purity profile, assay value and dissolution rates in unlimited number of monographs. It is precisely for these reasons that almost every laboratory today is equipped HPLC system.

However, it cannot be denied that more than often, the systems are working beyond their capacities and mostly dedicated who would like to change a well running stabilized column and prepare fresh solutions only because few assorted samples even though urgent are required to be analyzed.

1.6.10.1. Various Steps Involved in HPTLC

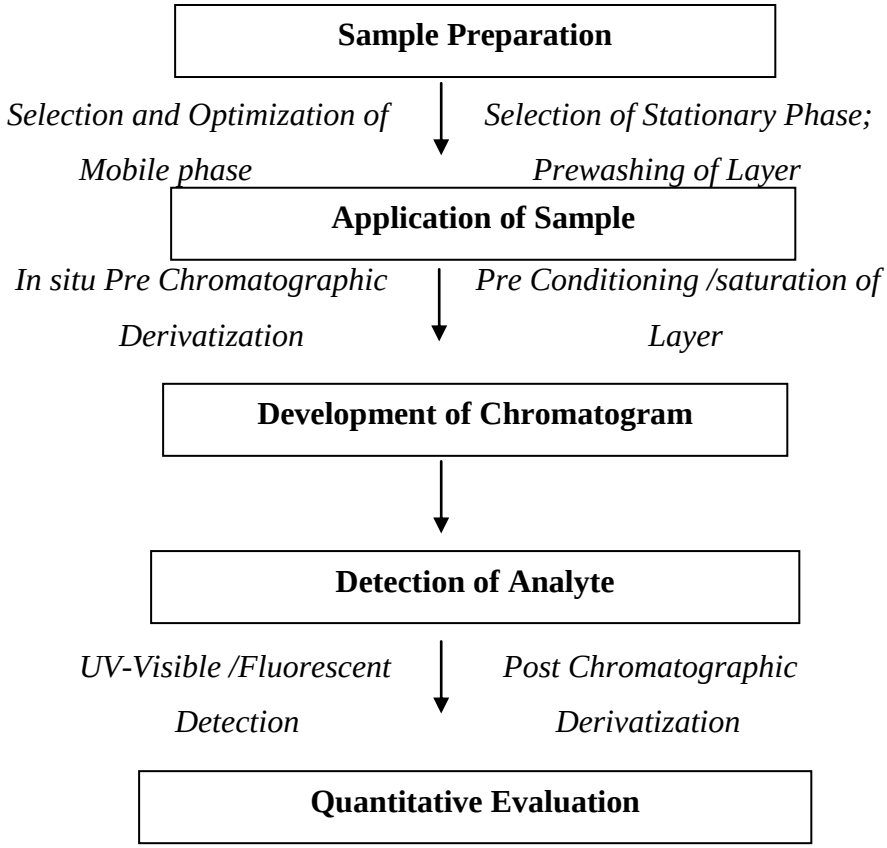
- Selection of HPTLC plates and sorbent.
- Samples preparation including any clean up and
- Pre-chromatographic derivatization.
- Application of sample.
- Development.
- Detection including post-chromatographic derivatization.
- Quantification.
- Documentation.

1.6.10.2. Criteria for Identification of an Analyte by HPTLC

- Rf value of an analyte should agree ± 3 % compared to standard material used under similar conditions.
- Visual appearance of the analyte should be indistinguishable from that of standard material.
- Centre of the spot nearest to that due to analyte should be separated from it by at least half the sum of analyte spot diameter.
- For conforming the identity, co-chromatography is mandatory, as a result, only the spot supposed to be due to analyte should be visible and no additional spot appear.
- Whenever spectrum detection is used, maximum absorption wavelength of the sample and standard should be same within limits of resolution of detection system and UV spectra should not be visibly different from that of the standard material.

1.6.10.3. HPTLC Method Development

Method Development for HPTLC



1.6.10.4. Factors Influencing the HPTLC Separation and Resolution of Spots

- Type of stationary phase, its particle size and activity
- Type of plates
- Layer thickness
- pH of the layer
- Binder in the layer
- Mobile phase
- Solvent purity
- Type and size of developing chamber
- Degree of chamber saturation
- Solvent for the sample preparation
- Sample volume spotted
- Size of initial spot and gradient
- Temperature and relative humidity

1.7. ANALYTICAL VALIDATION (PD Sethi *et al*., 1996)

Analytical validation of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety/efficacy throughout all phases of its shelf life. Such

monitoring is in accordance with the specifications elaborated/validated during the product development.

Analytical method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Method validation is the documented successful evaluation of an analytical method that provides a high level of assurance that such method will consistently yield result that are accurate with in previously established specification.

Analytical testing of a pharmaceutical product is necessary to ensure its purity, stability, safety and efficacy. Analytical method validation is an integral part of the quality control system.

Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones. Analytical method validation ensures that the selective analytical method will give reproducible and reliable results adequate for intended purpose.

1.7.1. INTRDUCTION TO VALIDATION

The word Validation means "Assessment" of validity or action of proving effectiveness. Various standardizing authorities defined the term validation as follows.

FDA defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes".

EUGMP define validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material, activity or system actually lead to expected result".

Australian GMP defines validation as "the action of proving that any material, process, activity, procedure, system, equipment or mechanism used in manufacture or control can and will be reliable and achieve the desire and intended result".

The objectives of the validation are,

- The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, quality, and purity they purport or are represented to possess.

- Assurance of Quantity.
- Government Regulation.

The validation is divided into different types. They are,

1. Prospective validation

This method is employed when historical data of the Product is not available or is not sufficient and in process and finished product testing is not adequate to ensure reproducibility or high degree of compliance to product likely attributes.

2. Retrospective validation

This provides trend of comparative result (i.e.) review and evaluation of existing information for comparison when historical data is sufficient and readily available.

3. Concurrent validation

Based on information generated during implementation of a system for this extensive testing and monitoring are performed as part of initial run of the method.

4. Re-validation

Revalidation provides the evidence that changes in a process and are the process environment, introduced either intentionally or unintentionally, do not adversely affect process characteristic and product quality. There are two basic categories of revalidation. Revalidation in case of known change. Periodic revalidation carried out at scheduled intervals.

1.7.2. Analytical Parameters used in Assay Validation as per ICH Guidelines
(Code Q2A; Q2B, ICH Guidelines 1994 and 1996; USP, 1995)
The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

1.7.3. Types of Analytical Procedures to be validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures,

1.7.3.1. Identification Tests

- Quantitative tests for impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.
- A brief description of the types of tests considered in this document is provided below.
- Identification tests are intended to ensure the identity of an analyte in a sample this is normally achieved by comparison of a property of the sample (example spectrum, chromatographic behaviour, chemical reactivity etc.) to that of a reference standard.

1.7.3.2. Accuracy

The word accuracy refers to term trueness. It expresses the closeness between the true value or the reference value and the value found in the analysis. True value is the accepted value of the reference value. The accuracy is determined by the recovery studies.

1.7.3.3. Precision

Precision expresses the degree of scatter between a series of measurement made in multiple sampling from the same homogenous sample. It may be considered fewer than three levels

- Repeatability
- Intermediate precision
- Reproducibility

The precision is expressed as Variance, standard deviation and coefficient of variation for a series of measurements.

The repeatability is confirmed by a minimum of 6 estimations at 100% of test concentrations. The standard deviations should be less than 2.

The intermediate precision is confirmed by inter day and intraday analysis, different instruments and different analyst.

1.7.3.4. Specificity

Specificity refers to the ability of the method to assess the analyte in the presence of other components like impurities, matrix or degradants, etc. The implications of specificity are

Identification: To ensure identity

Purity tests: To determine the content of impurity

Assay: Content of the analyte in the sample

1.7.3.5. Limit of Detection

It is the lowest amount of an analyte that can be detected by the analytical procedure but cannot be quantified exactly.

The LOD is performed based on the following parameters

- Based on visual examination
- Based on signal to noise ratio
- Based on the standard deviation and slope value.

The visual examination is done by analysing the sample with known quantity of standard and by establishing the minimum level at which the analyte can be detected.

A signal to noise ratio of 3 o 2.1 is considered as acceptable value for calculating the detection limit.

Based on slope and standard deviation values, Detection limit can be calculated by using the formula,

$$LOD = \frac{3.3\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve (of the analyte)

1.7.3.6. Limit of Quantitation

The lowest amount of the analyte which can be quantified by an analytical method with precision and accuracy is the limit of quantification.

Three approaches are made for determining the quantification limit. They are similar to that of determining the detection limit.

- Based on visual examination
- Based on signal to noise ratio
- Based on slope and standard deviation value

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

1.7.3.7. Linearity

Linearity is the ability of an analytical method to obtain results which are directly proportional to the analyte concentration within a given range. The linearity is evaluated as a plot of signals as a function of analyte concentration. The statistical parameters such as the slope, intercept, regression equation and correlation coefficient are calculated. For establishing of linearity, a minimum of 5 concentrations is required.

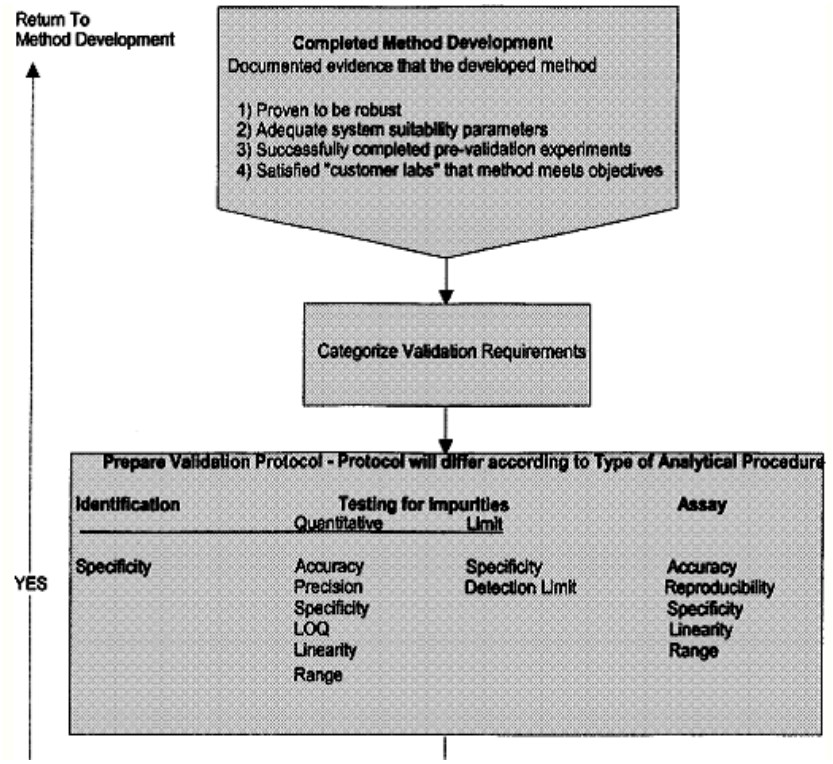
1.7.3.8. Range

It is the interval between the lower and upper limit of concentration in a sample for which the analytical method has suitable precision, accuracy and linearity. The minimum range considered for the assay of drug or finished product is form 80 to 120 percent of the test concentration.

1.7.3.9. Robustness

It is the ability of an analytical method to remain unaltered by small but deliberate variations in various parameters of the method and indicate its reliability. The typical variation includes stability of analytical solutions and extraction time. In case of HPLC, the change in the ratio of mobile phase, flow rate, variation of pH of the solution are done for determining the robustness of the method.

1.7.4. Complete Method Development and Validation Parameters



Acceptance criteria of validation

S.No.	Characteristics	Acceptance Criteria
1	Accuracy	Recovery 98-102% with 80,100,120% spiked sample.
2	Precision	RSD < 2%
2a	Repeatability	RSD < 2%
2b	Intermediate Precision	RSD < 2%
3	Specificity/ Selectivity	No interference
4	Detection Limit	S/N > 2 or 3
5	Quantification Limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80-120%

1.8. BASIC STATISTICAL PARAMETERS

(Gupta, *et al*., 1995; Bolton, *et al*.,2004; Mendham, *et al*., 1994)

Statistical techniques have been widely used in many diverse areas of scientific investigation. Statistical applications have been recognized as crucial to quality control procedure, test, specification and definitions. Principle of modern analytical techniques and skill in their application are necessary attribute of the successful pharmaceutical analyst, thus does not ensure the satisfactory solution of all the problem that may encountered. Some auxiliary knowledge methods those can aid the analyst in designing experiment, collecting data, and interpreting the result.

1.8.1 Linear Regression

Linear regression is a statistical technique that defines the functional relationship between two variables by best-fitting straight line. Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is

$$y = mx + c$$

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2} \quad \text{and} \quad c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

1.8.2 Correlation Coefficient (r)

It is a procedure commonly used to characterize quantitatively the relationship between variable. Correlation is related to linear regression. To establish whether there is a linear relationship between two variables x_1 and y_1**,** use Pearson's correlation coefficient r.

$$r = \frac{n\sum x_1 y_1 - \sum x_1 y_1}{\{[n\sum {x_1}^2 - (\sum x_1)^2]\,[n\sum {y_1}^2 - (\sum y_1)^2]\}^{1/2}}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative correlation

values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

1.8.3 Standard Deviation (SD)

It is commonly used in statistics as a measure of precision statistics as a measure of precision and is more meaningful than is the average deviation. It may be thought of as a root-mean-square deviation of values from their average and is expressed mathematically as

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x_i - \bar{x})}{N-1}}$$

Where,

S is standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the denomination is N -1 or N

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = deviation of a value from the mean.

N = Number of observations.

1.8.4 Percentage Relative Standard Deviation (%RSD)

It is also known as coefficient of variation (CV). It is defined as the standard deviation (SD) expressed as the percentage of mean.

$$CV \text{ or } \% \text{ RSD} = \frac{S.D}{\bar{x}} \times 100$$

Where,

SD = the standard deviation,

$\bar{x}$ = Mean or arithmetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

1.8.5 Standard Error of Mean (SE)

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observations. It is mathematically expressed as

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

Where,

SD = Standard deviation.

n = number of observation

1.8.6 Confidence Interval (CI)

A confidence interval gives an estimated range of values which is likely to include a unknown population parameter, the estimated range being calculated from a given set of sample data. A confidence interval with a particular confidence level (95% selected by the user) is intended to give the assurance that, if the statistical model is correct then the interval could deliver the true value.

Confidence interval for a normal population,

$$\bar{Y} \pm \frac{z_{\alpha/2}\ \sigma}{\sqrt{N}}$$

Where

$\bar{Y}$ = Sample mean

$z_{\alpha/2}$ = upper $\alpha/2$ critical value of standard normal distribution

N = Size of sample

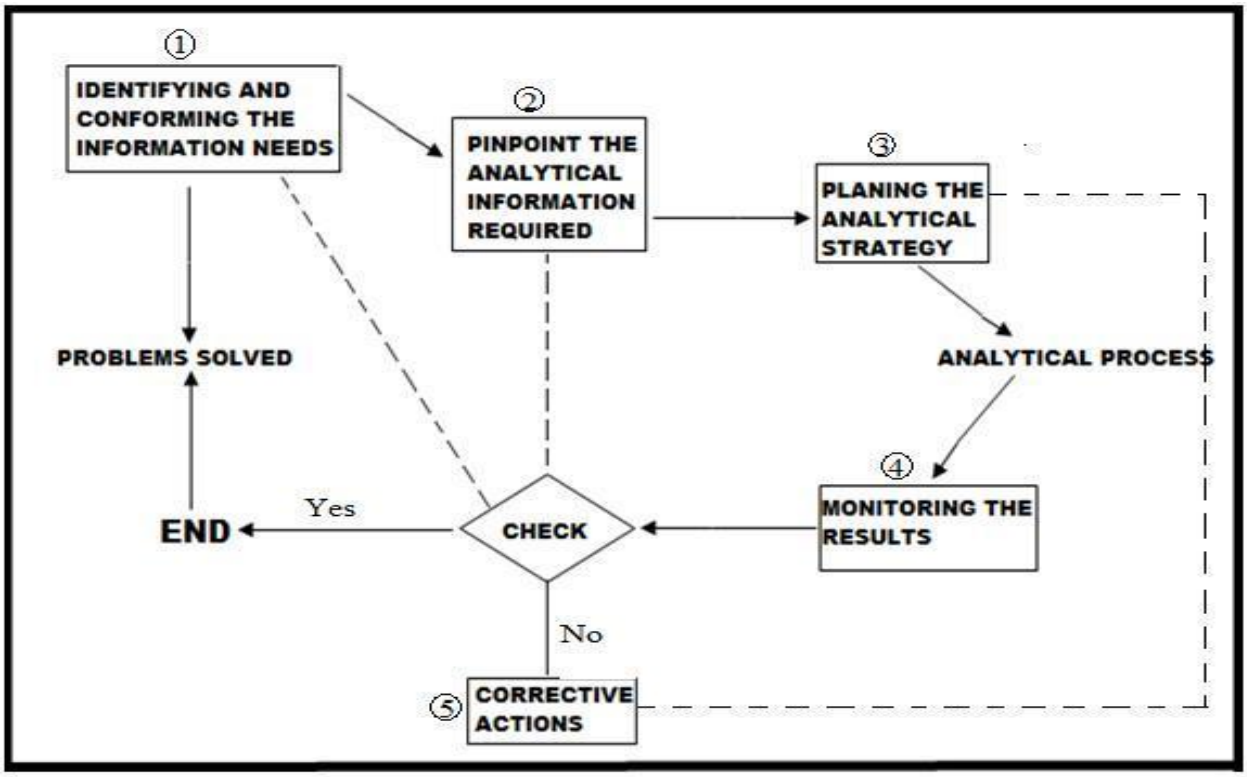
σ = Standard deviation

1.9. QUALITY AND THE ANALYTICAL PROBLEM

The general approach to quality encompasses both the basic and the practical side and their mutual complementary relationships. Analytical chemical quality has two additional components namely: (i) Metrological features, which include capital (accuracy, traceability, uncertainty) and basic analytical properties (precision, sensitivity, selectivity); and (ii) the analytical problem- solving process, which is related to capital and basic properties, and also to productivity- related properties (expeditiousness, cost- effectiveness, personnel-related factors). The basic and applied sides of quality in general and the analytical problem in particular are mutually related.

Properly solving analytical problems in order to ensure client satisfaction is thus an essential ingredient of analytical quality. At present, analytical quality is frequently identified almost soley with the metrological side. It is widely accepted that *"no quality is to be expected unless the objectives are properly defined"*; in fact, the analytical problem provides analytical chemists with such objectives. Also, *"quality is the concern of us all rather than a few"*. It is therefore essential to have analytical laboratories systematically plan and solve analytical problems if integral analytical quality is to be achieved; this entails breaking some traditional barriers of analytical chemistry.

Five General Steps Involving in Planning and Solving Analytical Problems



1.10. ANALYTICAL ERRORS (Kellner *et al*., 2004)

Analytical information can be classified in to three major groups according to its nearness to the true value. Further classification allows one to establish the so-called "Chemical metrological hierarchy"

Analytical errors can be defined as alterations of the analytical information delivered, i.e. as differences between the true values or held as true and the parameters on the other levels of the metrological hierarchy, and also as differences between results. An analytical error can arise both from an analytical result and analytical process. It can be expressed in absolute or relative terms. There are three main types of analytical errors are random error, systemic error and gross error.

1.10.1. Types of Analytical Errors

Differences between types of errors	**Types of Errors**		
	Random	**Systematic**	**Gross**
Source	Indeterminate	Determinate (well defined)	
References for definition	Means of a set (x□, μ')	True value (xˆ) value held as true (xˆ)	
Sign	Unpredictable (+) and (-)	Unique (+) or (-)	
Relative Magnitude (in general terms)	Small	Small	Large
Analytical properties involved	Precision	Accuracy	
		Uncertainty (traceability)	

LITERATURE REVIEW

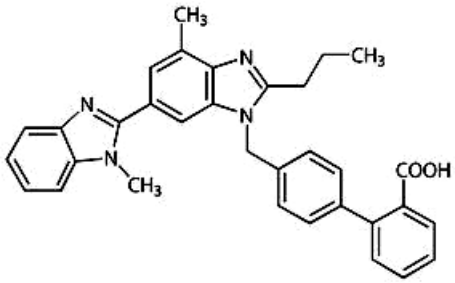
2. LITERATURE REVIEW

2.1 DRUG PROFILE

2.1.1 Telmisartan

(BP,2009; The Merck Index, 2006; Clarks Analysis of Drugs and Poisons, 2004; www.rx list.com; www.caymanchem.com/catalog/11615, www.guidechem.com)

Molecular Structure



Chemical Name

4′-[(1,4′-Dimethyl-2-propyl[2,6′-bi-1H-benzemidazole]-1′-yl)methyl]-[1,1′-biphenyl]-2-carboxylic acid.

Molecular Formula

$C_{33}H_{30}N_4O_2$

Molecular Weight

514.6

Category

Telmisartan is an angiotensin II receptor antagonist (ARB) used in the management of hypertension. Generally, angiotensin II receptor blockers (ARBs) such as telmisartan bind to the angiotensin II (AT1) receptors with high affinity, causing inhibition of the action of angiotensin II on vascular smooth muscle, ultimately leading to a reduction in arterial blood pressure.

Description

White or slightly yellowish, crystalline powder.

Solubility

Soluble in strong base, sparingly soluble in strong acid (except hydrochloric acid), sparingly soluble in methylene chloride, slightly soluble in methanol, practically insoluble in water.

Identification

1. Melting Point

Standard Value	Observed Value*
261º - 263ºC	262.3ºC

(*Average of six observations)

2. IR spectrum was recorded and shown in figure 1

Storage

Stored in cool dry place (25^0 C), the bottles should be tightly closed

pKa value

3.83

Pharmacology Description

Telmisartan is an angiotensin II receptor antagonist (ARB) used in the management of hypertension. Generally, angiotensin II receptor blockers (ARBs) such as telmisartan bind to the angiotensin II type 1 (AT1) receptors with high affinity, causing inhibition of the action of angiotensin II on vascular smooth muscle, ultimately leading to a reduction in arterial blood pressure. Recent studies suggest that telmisartan may also have PPAR-gamma agonistic properties that could potentially confer beneficial metabolic effects.

Mechanism of Action

Telmisartan interferes with the binding of angiotensin II to the angiotensin II AT_1-receptor by binding reversibly and selectively to the receptors in vascular smooth muscle and the adrenal gland. As angiotensin II is a vasoconstrictor, which also stimulates the synthesis and release of aldosterone, blockage of its effects results in decreases in systemic vascular resistance. Telmisartan does not inhibit the angiotensin converting enzyme, other hormone receptors, or ion channels. Studies also suggest that telmisartan is a partial agonist of PPARγ, which is an established target for antidiabetic drugs. This suggests that telmisartan can improve carbohydrate and lipid metabolism, as well as control insulin resistance without causing the side effects that are associated with full PPARγ activators.

Pharmacodynamic

Telmisartan is an orally active nonpeptide angiotensin II antagonist that acts on the AT1 receptor subtype. It has the highest affinity for the AT1 receptor among commercially available ARBS and has minimal affinity for the AT2 receptor. New studies suggest that telmisartan may also have PPARγ agonistic properties that could potentially confer beneficial

metabolic effects, as PPARγ is a nuclear receptor that regulates specific gene transcription, and whose target genes are involved in the regulation of glucose and lipid metabolism, as well as anti-inflammatory responses. This observation is currently being explored in clinical trials. Angiotensin II is formed from angiotensin I in a reaction catalyzed by angiotensin-converting enzyme (ACE, kininase II). Angiotensin II is the principal pressor agent of the renin-angiotensin system, with effects that include vasoconstriction, stimulation of synthesis and release of aldosterone, cardiac stimulation, and renal reabsorption of sodium. Telmisartan works by blocking the vasoconstrictor and aldosterone secretory effects of angiotensin II.

Pharmacokinetics

Absorbtion

Absolute bioavailability depends on dosage. Food slightly decreases the bioavailability (a decrease of about 6% is seen when the 40-mg dose is administered with food). Rapidly absorbed. Dose dependent bioavailability 42% (after 40 mg dose); 58% (after 160-mg dose). Peak plasma concentrations: 0.5-1 hours.

Distribution

Telmisartan is more than 99.5% protein bound.

Metabolism

Minimally metabolized by conjugation to form a pharmacologically inactive acylglucuronide; the glucuronide of the parent compound is the only metabolite that has been identified in human plasma and urine. The cytochrome P450 isoenzymes are not involved in the metabolism of telmisartan.

Elimination

Either intravenous or oral administration of telmisartan, most of the administered dose (>97%) was eliminated unchanged in feces via biliary excretion; only minute amounts were found in the urine (0.91% and 0.49% of total radioactivity, respectively).

Adverse Effects

Upper respiratory track infection, dizziness, back pain, sinusitis, pharyngitis and diarrhoea. Slight elevations in liver enzymes.

Potentially Fatal: Rarely angioedema, rash, pruritus and urticaria.

Drug Interactions

Acetyl salicylic acid, amiloride, celecoxib, diclofenac, diflunisal, fenoprofen, drospirenone, flurbiprofen, ketoprofen above the drugs interacts with telmisartan, may increase the risk of acute renal failure and hyperkalemia.

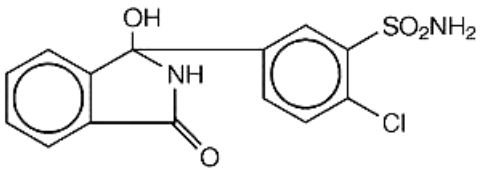
Toxicity

Intravenous LD_{50} in rats is 150-200 mg/kg in males and 200 to 250 mg/kg in females. Acute oral toxicity is low: no deaths and no changes occurred in rats or dogs at 2000 mg/kg, the highest dose tested. Limited data are available with regard to overdosage in humans. The most likely manifestations of overdosage with telmisartan would be hypotension, dizziness and tachycardia; bradycardia could occur from parasympathetic (vagal) stimulation.

2.1.2 CHLORTHALIDNE

(*BP, 2009; USP, 2009; Martindale The Extra Pharmacopeia, 1993; The Merck Index, 2006. Analytical Profile of Drug Substance, 2005.)*

Molecular Structure



Chemical Name

2-chloro - 5 (2, 3, dihydro,-1-hydroxy-3-oxo-1H-isoindol-1-yl) benzene sulphonamide.

Molecular Formulae

$C_{14}H_{11}Cl\ N_2O_4S$

Molecular Weight

338.77 g/ mol

Category

Chlorthalidone is diuretic, it indicated in the management of hypertension as sole therapeutic agent or in combination with other antihypertensive drugs. It is used as adjunctive therapy in the treatment of edema associated with heart failure, hepatic cirrhosis. It has also been found useful in the edema due to various forms of renal dysfunction such as nephrotic syndrome.

Chlorthalidone has been used in the treatment of premenstrual tension if there is evidence of fluid retention. It may prevent renal calculus formation in patients with hypercalciuria.

Description

White or yellowish-white crystalline powder.

Solubility

Practically insoluble in water, in ether, and in chloroform; slightly soluble in in alcohol; soluble in methyl alcohol and in acetone; soluble in solutions of alkali hydroxides.

Identification

1. Melting Point

Standard Value	Observed Value*
218^{o}-220ºC	218ºC

(* Average of six observations)

2. IR spectrum was recorded and shown in figure.2

Storage

Stored in well closed container below 40° C

pKa value

9.4

Mechanism of Action

Chlorthalidone inhibits sodium ion transport across the renal tubular epithelium in the cortical diluting segment of the ascending limb of the loop of Henle. By increasing the delivery of sodium to the distal renal tubule, Chlorthalidone indirectly increases potassium excretion via the sodium-potassium exchange mechanism.

Pharmacodynamics

The diuretic effect of the drug occurs within two hours after an oral dose and continues for up to 72 hours. It produces copious loss of electrolytes, and consequently, of water. The site of action is the distal renal tubul. The hypotensive effect is also due to a reduction in peripheral resistance observed mainly in chronic use.

Pharmacokinetics

Absorbtion

Chlorthalidone is erratically absorbed from the gastrointestinal tract. Bioavailability after oral administration is approximately 65% peak blood concentrations being attained after 8 to 12 hours. Chlorthalidone crosses the placental barrier and passes into breast milk

Distribution

The volume of distribution of chlorthalidone is 3.9 ± 0.8 l/ kg About 75% is bound to plasma protein and the blood-to-plasma ratio is 72.5%. And crosses the placenta.

Metabolism

Metabolized in Liver

Elimination

During long-term administration, 30 to 60% has been reported to be excreted unchanged in the urine. The urinary excretion after 50 and 100 mg doses. The clearance of chlorthalidone is 1.6 ± 0.3 ml/ min/ kg, and decreases with age and at greater doses.

Chlorthalidone is excreted in breast milk.

Adverse Effects

Hypotension, Vasculitis, Electrolytes abnormal, Hyperglycemia, Hyperuricemia, Diarrhea, Loss of appetite, Nausea, Vomiting, Dizziness, Headache, Paresthesia, Cardiac dysrhythmia (rare), Pulmonary edema (rare).

Drug interactions

Deslanoside, diazoxide, digitoxin, digoxin, dofetilide, lithium, tenoxicam, trandolapril, treprostinil

Toxicity

Symptoms of overdose include nausea, weakness, dizziness and disturbances of electrolyte balance.

2.2. REPORTED METHODS

1. AjitPandey*et al*., (2011), reported **"UV- Spectrophotometric method for estimation of Telmisartan in bulk and tablet dosage form".** UV Spectrophotometric method has been developed and validated for the estimation of Telmisartan in bulk and tablet dosage form. The zero order spectra of telmisartan in 0.1 M sodium hydroxide shows λmax at 234.0 nm calibration graph was found to be linear over the concentration range 4-24μg/ ml.

2. AkifulHaque.M*et al*., (2012), reported **"Simultaneous Estimation of Atenolol and Chlorthalidone as Bulk and in Tablet dosage Form Using UV-spectrophotometry".** UV Spectrophotometric method has been developed for the simultaneous estimation of atenolol and Chlorthalidone in bulk and its combined tablet dosage form. The stock solutions were prepared in methanol followed by further required dilution with methanol. The absorbance maxima of atenolol and Chlorthalidone were found to be 225 nm and 284 nm respectively. Beer law obeyed the concentration range of atenolol is 10-60 μg/ ml and chlorthalidone is 30-140 μg/ ml .

3. AshaB.Thomas*et al*., (2010), reported **"SimultaneousSpectrohotometric Estimation of Amlodipine Besylate and Telmisartan in Tablet Dosage Form".**First method involves determination using the simultaneous equation method, the sampling wavelength selected are 364.5 nm and 252.5 nm over the concentration range of 1-50 μg/ ml and 10-50 μg/ ml for Amlodipine besylate and Telmisartan, respectively. The second method is the Area under Curve method, the sampling wavelength range selected are 366.5-362.5 nm and 256.5-252.5 nm with the linearity concentration range of 1-50 μg/ ml and 10-80 μg/ ml for Amlodipine besylate and Telmisartan respectively. The third method using the multicomponent mode method, the sampling wavelength selected are 364.5nm and 254.5 nm over the concentration range of 1-0 μg/ ml and 10-80 μg/ ml for Amlodipine besylate and Telmisartan respectively.

4. Bankey S *et al*., (2009), reported **"Simultaneous Determinaton of RamiprilHydrochlorthizide and Telmisartan by Spectrophotometry".**The multicomponent mode analysis method has been developed for simultaneous determination of ramipril,hydrochlorothiazide and telmisartan in tablet formulation. The wavelength selected for these drugs were 218 nm, 271 nm, and 296 nm, respectively using methanol as

solvent.the linearity for these drug at all the selected wavelength lies between 0.5-3.5 µg/ ml for Ramipril, 1.25-8.75 µg/ ml for Hydrochlorthiazide and 4-28 µg/ ml for Telmisartan.

5. BhaskaraRaju*et al*., (2011), reported **"Novel HPLC Method Development Validation and Estimation of Telmisartan in Bulk and Its Pharmaceutical Formulation".** Separation of drug was achieved on reverse phase C_8 column using acetonitrile and phosphate buffer (pH 3.0) as mobile phase in the ratio of 60:40 v/v, with the flow rate of 0.9 ml/ min at 229 nm as detection wavelength the linearity was observed in the range of 20-60 µg/ ml with correlation coefficient of 0.9996.

6. Bhatia NM.*et al*., (2010), reported **"Development and Validation of Spectrophotometric and Ion Pair Chromatographic Techniques for Estimation of Telmisartan and Hydrochlorothiazide".** The first method involves multiwavelength spectrophotometric estimation where interference due to hydrochlorothiazide at 286 nm (wavelength for the estimation of telmisartan) and 308 nm whereas interference of telmisartan at 262 nm (wavelength for the estimation of hydrochlorothiazide) was removed by recording absorbance difference at 262 nm and 282 nm. Linearity of the response was demonstrated by telmisartan in the concentration range of 5-35 µg/ ml and the concentration range of 3-21 µg/ ml for hydrochlorothiazide with correlation coefficient of 0.9995 and 0.9992 for telmisartan and chlorothiazide. The second method utilize ion pair chromatography on a HIQ Sil ODS column, using mobile phase as Methanol: 0.0025M orthophosphoric acid in the ratio 70:30 v/v at pH 4.6 containing 0.1% 1- hexane Sulphonic acid monohydrate sodium salt as mobile phase with UV detection at 259 nm over a concentration range of 20-120 µg/ ml for telmisartan and 12.5-75 µg/ ml for hydrochlorothiazide, Losartan Potassium was used as the internal standard.

7.Brijesh Singh*et al*., (2009), reported **"A Reversed- Phase High Performance Liquid Chromatographic Method for Determination of Chlorthalidone in Pharmaceutical Formulation".** The method was carried out on reverse phase C_{18} column using mixture of Acetonitrile, Methanol, and 50mM dihydrogenphosphate in the ratio of 05:30:70 v/v, pH adjustedto 3.5 with orthophosphoric acid as mobile phase. Hydrochlorothiazide was used as international standard. The detection was carried out by UV- Detector at 220 nm. The calibration was found tobe linear in the range of 0.1to3.2 µg/ ml.

8. Elshanawane AA*et al*., (2009), reported **"Development and Validation of a Reversed – Phase High-Performance liquid chromatographic method for the simultaneous determination of amiloride hydrochloride, atenolol, hydrochlorothiazide and chlorthalidone in their combined mixtures".** The method was developed for the simultaneous determination of 2 ternary mixtures containing amiloride hydrochloride, atenolol, hydrochlorothiazide and chlorthalidone usingCyanopropyl column, the mobile phase consisted of 10mM KH_2PO_4 Buffer pH 4.5 and Methanol in the ratio of 75:25 % v/v, at the flow rate 1 ml/min. Uv detector was operate 275 nm. Calibration graphs were linear in the concentration ranges of 2-10µg/ml, 20-200 µg/ ml, 10-100 µg/ ml and 5-50 µg/ ml for amiloride hydrochloride, atenolol, hydrochlorothiazide and chlorthalidone, respectively.

9.Faimida Jahan *et al*., (2012), reported **"Simultaneous Estimation of Telmisartan and Cilinidipine in solid dosage form".** Spectrophotometric method (Vierodts method), was developed and validated for the determination of cilinidipine and telmisartan in tablet dosage form. In developed method cilinidipine and telmisartan were quantified using their absorptivity values at selected wavelength,viz., 350 nm and 294 nm respectively.

10.Jat R.K. *et al*., (2012), reported **"Quantitative Estimation of Telmisartan in Bulk Drug and Tablets by UV Spectroscopy".**Spectrophotometer method has been developed for the assay of Telmisartan in bulk drugs and tablets. Telmisartan shows maximum absorbance at 216 nm. Beer's law was obeyed in the concentration range of 5-25 µg / ml. With correlation coefficient of 0.999.

11. Kalyan Kumar B. *et al*., (2011), reported **"Development and Validation of RP-HPLC Method for Simultaneous Estimation of Ramipril, Telmisartan and Hydrochlorothiazide in Pharmaceutical dosage Forms".** Isocratic high performance liquid chromatographic method was developed and validated for the determination of Hydrochlorothiazide, Ramipril and Telmisartan in tablet formulation. The method employs waters HPLC system on XTerra RP_8 column and flow rate 0.8 ml/ min with load of 20 µl. Acetonitrile and Phosphate buffer (pH 3.0) was used as mobile phase in the composition 45:55 the detection was carried out at 215 nm. Linearity ranges for Hydrochlorothiazide, Ramipril and Telmisartan were 12.5-22.5 µg /ml, 5-9 µg/ ml, and 40-72 µg / ml respectively. Retention time of Hydrochlorothiazide, Ramipril, Telmisartan were found to be 2.83, 3.65 and 5.03min, respectively.

12.Kondawar M.S. *et al.*,(2011), reported **"UV Spectrophotometric Estimation of Amlodipine besylate and Telmisartan in Bulk Drug and Dosage Form by Multiwavelength Analysis".** The Spectrophotometric method has been developed for the simultaneous estimation amlodipine besylate and telmisartan in combined table dosage forms. The tablet is determined by the multi-wavelength technique at the wavelength of 360 nm and 298 nm over the concentration ranges of 15-75 μg/ ml and 1-10 μg/ ml using methanol as solvent.

13. Kumbhar S.T. *et al*., (2011), reported **"Visible Spectrophotometric Determination of Telmisartan from Urine".** This method can find application in clinical studies and therapeutic drug monitoring. This method of colorimetric estimation of yellow coloured chromogen when its react with alizarin in presence of thionyl chloride. The concentration of telmisartan over a range of 10-60 μg/ ml. The yellow coloured complex has absorption maxima at 427 nm.

14. Kumar G.S. *et al*., (2012), reported **"Development and Validation of RP-HPLC method for Simultaneous Estimation of Atenolol and Chlorthalidone from Pharmaceutical Formulation".** A HPLC method has been developed and validated for the simultaneous estimation of Atenolol and Chlorthalidone in marketed formulation. The determination was carried out on an XTerra RP column using a mobile phase of containing Methanol: Potassium dihydrogen Phosphate buffer (50:50v/v adjusted to pH 3.4) with flow rate 0.5ml/ min UV detection at 240 nm. The retention time for Atenolol was 3.2 min and for Chlorthalidone 5.0 min. Atenolol and Chlorthalidone showed a linear response in the concentration range of 50-150 μg/ ml. The correlation coefficient for Atenolol and Chlorthalidone was 0.9996.

24. Kurade V.P. *et al*., (2009), reported **"RP-HPLC Estimation of Ramipril and Telmisartan in Tablets".** The separation was achieved by Genesis C_{18} column having dimensions of 4.6×250 mm and particle size of 5 μm in isocratic mode, with mobile phase containing a mixture of 0.01 M potassium dihydrogen phosphate buffer (adjusted to pH 3.4 using ortho phosphoric acid): methanol : acetonitrile (15:15:70 v/v/v) was used. The mobile phase was pumped at a flow rate of 1.0 ml/ min and the eluents were monitored at 210 nm. The selected chromatographic conditions were found to effectively separate Ramipril (R_t: 3.68 min) and Telmisartan (R_t: 4.98 min) having a resolution of 3.84.

16. Lakshmi KS*et al*., (2010), reported **"Design and Optimization of a Chemometric-Assisted Spectrophotometric Determination of Telmisartan and Hydrochlorothiazide in Pharmaceutical Dosage Form".** The chemometric methods applied were principle component regression and partial least square. These approaches successfully applied to quantity the two drugs in the mixture using the information include in the UV absorption spectra of appropriate solution in the range of 200 – 350 nm.

17. Leena R. Bhat*et al*., (2007), reported **"Validated RP-HPLC Method for the Simultaneous Determination of Telmisartan and Hydrochlorothiazide in Pharmaceutical Formulation".** The mobile phase consisted of Acetonitrile: Methanol (30:70 v/v) at flow rate 1ml/ min and wavelength detection was 270 nm. Rabeprazole was used as an internal standard. The retention time for telmisartan, hydrochlorthiazide and rabeprazole were 1.79, 2.80 and 3.19 minutes, respectively.

18. Luz Luis M. *et al.*,(1999),reported **"Simultaneous Determination of Chlorthalidone and Spironolactone with Univariate and Multivariate Calibration: Wavelength Range Selection "**.Univariate Calibration was performed by the Zero-crossing and Derivative ratio spectrum methods. Extensive spectral overlap and the Scarcityo of wavelength in derivative spectra allowing one analyte to be distinguished and quantitated in the presence of the other gave rise to poor results that called for multivariate calibration.

19.MadhuBabuKasimala. *et al*., (2012), reported **"Reverse Phase –HPLC Method Development and Validation for the Simultaneous Estimation of Azilsartanmedoxomil and chlorthalidone in pharmaceutical Dosage Forms".**The method is isocratic elution at flow rate of 0.9ml/ min was employed on a summary C_{18} column at ambient temperature. The mobile phase consisted acetonitrile; methanol: water: 0.1% orthophosphoric acid at the ratio of 15:30:15:5 (v/v/v/v). The UV detection wavelength was 251nm. The retention time for Chlorthalidone was 3.923 min and Azilsartanmedoxomil was 7.208 min.

20. Mayurmodi.*et al*., (2012), reported **"Development and Validation of Spectrophotometric Methods for simultaneous Estimation of Metoprolol succinate and Telmisartan in Combined Pharmaceutical Formulation".** Four spectrophotometric methods have been developed for simultaneous analysis of Metoprolol succinate and Telmisartan in Combined Pharmaceutical Formulation. Simultaneous equation method employs using two wavelengths 230.2 nm for metoprolol succinate, 237 nm for telmisartan.

First derivative Q-absorbance equation method. It involves, formation of Q-absorbance equation at 231.8 nm is absorptive point, 231.8 nm for Telmisartan in first derivative method. Absorbance correction method, involves measurement of corrected absorbance at 296.6 nm for Telmisartan and measurement of corrected absorbance at 223 nm for metoprolol succinate. Combination of first derivative dual wavelength, which uses the difference in absorbance at 282.4 nm 284.6 nm for estimation of Metoprolol succinate for zero crossing and 330 nm for estimation of Telmisartan in first derivative spectra. The calibration graph follows beer's law in the range of 3-20 µg/ ml for Metoprolol succinate and 4-16 µg/ ml for Telmisartan with R square value greater than 0.999.

21. Mhaske R A *et al*., (2012), reported **"RP-HPLC Method for Simultaneous Determination of Amlodipine , Valsartan, Telmisartan, Hydrochlorothiazide and Chlorthalidone: Application to Commercially available Drug Products ".** The separation was achieved on Cosmosil PAQ 4.6×150 mm and particle size of 5 µmcolumn with gradient flow.The mobile phase at a flow rate of 1ml /min consisted of 0.05M Sodium dihydrogen phosphate buffer and acetonitrile (gradient ratio) The UV detection was carried out at 220nm.

22. Mhaske R A *et al*., (2012), reported **"RP-HPLC Method for Simultaneous Determination of Atrovastatin Calcium, olmisartan medoxomil, Candesartan, Hydrochlorthiazide and Chlorthalidone : Application to Commercially available Drug Products ".** The separation was achieved on Cosmosil PAQ 4.6×150 mm and particle size of 5 µm column with gradient flow. The mobile phase at a flow rate of 1ml /min consisted of 0.05M Sodium dihydrogen phosphate buffer and acetonitrile (gradient ratio) The UV detection was carried out at 220 nm.

23. Niranjan D. Chivate*et al*., (2012), reported **"Development of UV Spectrophotometric Method for Estimation and Validation of Telmisartan as a Pure API".** The spectrophotometric method wavelength was selected 240 nm by using 60% ethanol (95%) and 40% of 0.1M $NaHCO_3$ as a solvent for Telmisartan. The linearity for this drug lies between 2-24 µg / ml.

25. Palled M S *et al*., (2006), reported **"Difference spectrophotometric Determination of Telmisartan in Tablet dosage Forms".** Spectrophotometric method was developed. Telmisartan exist in two different forms in acidic and basic medium that differ in their UV

spectra. Difference spectrum, obtained by keeping Telmisartan in 0.01N NaOH in reference cell and Telmisartan in 0.01N HNO_3 in sample cell, showed two characteristic peaks at 295 nm and 327 nm with positive negative absorbance respectively. The method was linear in the range of 2 -12 µg/ ml.

26. Prajakta S. Nawale*et al*., (2012), reported **"Normal and RP-HPTLC Methods for Simultaneous Estimation of Telmisartan and Metoprolol Succinate in Pharmaceutical Formulation".** Method I was developed with aluminium plates precoated with silica gel 60 F254S, and toluene: propanol: methanol: triethylamine (8:1:1: 0.5 v/v) was used as as mobile phase. Method II was carried out using aluminium coated with RP_{18} silica gel 60 F254S HPTLC plates using methanol : water : triethylamine (6 : 4 : 0.5 v/v) as mobile phase. Both analyses were scanned with a densitometer at 242 nm. In Method I, good separation and resolution of drugs were achieved with Rf values 0.45 ± 0.02 (Telmisartan) and 0.70 ± 0.02 (Metoprolol), while in Method II, Telmisartan and Metoprolol showed Rf values 0.55 ± 0.02 and 0.41 ± 0.02, respectively.

27. Patil U P *et al*., (2009), reported **"Simultaneous Determination of Atrovastatin Calcium and Telmisartan in Tablet Dosage form by Spectrophotometry".** Three Spectrophotometric methods were developed. Absorbance correction method employs wavelength 328 nm for direct estimation of Telmisartan where Atrovastatin nil absorbance. Estimation of Atrovastatin is carried out after correction for absorbance of Telmisartan at 241nm. Second method is based on First order derivative spectroscopy wavelengths 297 nm and 241.8 nm were selected for the estimation of atrovastatin calcium and telmisartan. Third method is based on dual wavelength method Atrovastatin calcium was determined by plotting difference in absorbance at 258 nm and 291nm (difference in zero for Telmisartan). Similarly Telmisartan the difference absorbance at 225 nm add 252 nm (Difference is zero for Atrovastatin calcium). Both the drug obeys beer's law in the concentration range 5-30 µg / ml.

28. Popat B Mohite*et al*., (2010), reported **"Simultaneous Estimation of Ramipril and Telmisartan in Tablet Dosage Form by Spectrophotometry".** The multicomponent method it involves absorbance measurement at 205 nm and 291 nm for Ramipril and Telmisartan in 0.2M H_2SO_4. Beers law obeys the concentration range of 5-40 µg/ ml for Ramipril and 2-20 µg/ ml for Telmisartan. Graphical absorbance which is based on

measurement of absorbance of ramipril and telmisartanat 222 nm (isoabsorptive point of Ramipril and Telmisartan) and 291 nm.

29. Pratap Y Pawar*et al*., (2012), reported **"Simultaneous Spectrophotometry Estimation of Amlodipine Besylate and Telmisartan in Tablet Dosage Form".** The Simultaneous equation method is based on measurement of absorbance at 367 nm and 292 nm as two wavelengths selected for quantification of Amlodipine Besylate and Telmisartan. The First order derivative based on the measurement of absorbance at 270 nm and 295 nm as two wavelengths selected for quantification of Amlidipine Besylate and Telmisartan. Both method obey Beer's law in the concentration range of 20-100 µg/ ml for A and 5-30 µg /ml for Telmisartan.

30. Ramesh L Sawant *et al*., (2012), reported **"Validated Spectrophotometric Method for Simultaneous Estimation of Telmisartan and indapamide in pharmaceutical Dosage Form".** The absorption maxima were found to be at 296 nm and 242 nm in methanol for telmisartan and intapamide respectively. Beer's law obey in the concentration range of 5-25 µg/ ml for Telmisartan and 10-30 µg/ ml for indapamide with correlation coefficient within the range of 0.996-0.998 for both drug. The simultaneous equation method is based on the multi component analysis involves recording absorbance of standard solution at 296 nm and 242 nm.

31. Ridhdhi S Sinojiya *et al*., (2012), reported **"Development and Validation of RP-HPLC Method for the Simultaneous Determination of Telmisartan, Amlodipine Besylate and Hydrochlorothiazide in a Tablet Dosage Form".**Separation of Chromatographic method by using RP-C_{18} (Hypercil, 250× 4.6 mm), mobile phase Acetonitrile : 1% triethlyamine in ratio of 50 : 50 (pH adjusted to 3.5 with orthophosphoric acid) with flow rate of 1.2ml /min, injection volume 20 µL. Isocratic elution with total run time 8 min. detection of multi component was carried out at 237 nm. Retention time for Amlodipine, Hydrochlorothiazide, and Telmisartan was found to be 2.323, 2.810 and 5.527 min respectively. The calibration curve concentration range of 2-22.5 µg/ ml for Amlodipine, 6.25-56.25 µg/ ml for Hydrochlorothiazide and 20-180 µg/ ml Telmisartan. had regression coefficient greater than 0.999 for the all drugs.

32. Sagar Tatane., (2011), reported **"Development of UV Spectrophotometric Method of Telmisartan in Tablet Formulation".** The wavelength selected for the telmisartan was

230 nm. The linearity for the drug at the selected wavelength is lies between 1-8 µg/ ml, correlation coefficient - 0.999.

33. Santhos V Gandhi *et al*., (2011), reported **"A Validated Reverse Phase HPLC Method for Simultaneous Determination of Telmisartan and Ramipril as Bulk Drug and in Tablet dosage Form".**Separation of drug was carried out with HiQ-Sil C_8 column (250 mm × 4.6 mm) using Acetonitril:0.01 M Heptane sulphonic acid sodium salt (pH 2.8) (60:40 v/v) as mobile phase. The method was development using Phenylpropanolamine hydrochloric acid as internal standard. Detection at 216 nm. Beer's law obeyed the concentration range of 10-50 µg/ ml and 2.5-12.5 µg/ ml for Telmisartan and Ramipril respectively.

34. SubhakarNandipati *et al*., (2012), reported **"Development and Validation of RP-HPLC Method for Estimation of Telmisartan in Bulk and Tablet Dosage Form".** Separation of drug was carried out with C_{18} sun fire column (250 mm × 4.6 mm) 5µm. Mobile phase Acetonitril: Potassium di hydrogen phosphate (60:40 v/v) pH adjust with ortho phosphoric acid. Flow rate was 1ml/ min, Retention time was 3.4 for Telmisartan.

35. Sunil Jawla *et al*., (2010), reported **"Development and Validation of Simultaneous HPLC Method for Estimation of Telmisartan and Ramipril in Pharmaceutical Formulations".** Separation of drugs were carried out with ODS C_{18} column (250 mm × 4.6 mm) 5µm. using a mobile phase consisting of Acetonitrile:Potassium dihydrogen phosphate buffer having pH 2.8 in the ratio of 40: 60 v/v with the flow rate 1.5ml/ min using PDA detection at 210 nm. The linear response was observed over the concentration range of 2.5-25.5 ppm and 3.0-7.25 ppm for assay of Telmisartan and Ramipril respectively. The retention time for Telmisartan and Ramipril 5.7 min and 10.8 min respectively.

36. Sunil Singh., (2012), reported "**Simultaneous Eestimation of Telmisartan and Ramipril in Combined Dosage Form by using HPTLC".** The method was carried out in TLC Precoated silica gel on aluminum plate 60 F 254, (10 cm ×10 cm, prewashed by methanol and activated at 60° C for 5 min prior to chromatography). The solvent system was Acetone: Benzene: Ethyl acetate: Glacial acetic acid in the proportion of 6:4:1:0.05, (v/v/v/v) with Rf value for telmisartan and ramipril was0.673 and 0.353 respectively. The linearity regression analysis for calibration showed 0.999 and 0.998 for telmisartan and ramipril with

respect to peak area and height in the concentration range of 150- 1700 ng/spot and 300-1900 ng/ spot respectively.

36. Vijayamirthraj R *et al*., (2010), reported **"Development and Validation of RP-HPLC Method for Simultaneous Estimation of Telmisartan and Atrovastatin Calcium inTablet Dosage Form".** Separation of drugs were carried out with Phenomenex C_{18} column (250 mm × 4.6 mm) 5µm. using a mobile phase consisting of Acetonitrile: Potassium dihydrogen phosphate buffer (0.01 M) having pH 4 (adjusted with orthophosphoric acid) in the ratio of 65 : 35 v/v with the flow rate 2.0 ml/ min using PDA at 250nm.

37. Vijaya Kumar G *et al*., (2011), reported **"Validated of RP-HPLC Method for the Estimation of Telmisartanin Serum Samples".** Isocratic elution at a flow rate of 1.0 ml/ min was employed on a Equsil, 250×4.6mm, 5µ. The mobile consist of Acetonitrile: buffer (65: 35) v/v. the detection wavelength 282 nm and Retension time for telmisartan was 3.32 min.

38. Yogesh Gupta *et al*., (2009), reported **"Isocratic RP-HPLC-UV Method Development and Validation for the simultaneous Estimation of Ramipril and Telmisartan in Tablet Dosage Form".** Separation was carried out by using mobile phase Acetonitrle: Buffer (65:35) at pH 4.5 the flow rate 1.0ml/ min, Detection at 210 nm and Retention time for Ramipril and Telmisartan were4.25 min and 7.99 min respectively.

AIM AND
PLAN OF WORK

3. AIM AND PLAN OF WORK

3.1 AIM OF WORK

Hypertension is a chronic disorder very commonly seen among people. Current hypertension treatment guidelines recommend a goal of <140/90 mmHg for population with uncomplicated hypertension and goals are even lower (< 130/80 mmHg) for patients with diabetes or renal disease. These recommendations are supported by long-term trials suggesting that the greater reduction in blood Pressure, the greater reduction in risk of cardiovascular events. Major clinical studies have shown that most patients require two or more drugs to achieve their blood pressure goals. Combination therapy should be used as initial treatment for patients in whom the probability of achieving BP control with monotherapy is low. Given the number of antihypertensive agents available, the number of potential combinations is large. However, rational choices should be based on some requirements.

The prevalence of cardiovascular disease will undoubtedly increase as the mean age of the populations throughout the world increases. Heart disease is generally complex and multi factorial. Despite considerable advances in therapy, the underlying mechanisms are still rather poor. Efforts are directed towards the development of new drugs through the modification of the physicochemical properties of drug molecule, and the design and synthesis of new drugs with particular emphasis on drug delivery systems, pharmaceutical materials research, controlled drug release and targeting of drugs to the site of action to enhance the therapeutic effects. The perspective of the user enables the pharmacist and clinicians in understanding the factors leading to the suboptimal use of drugs in society. Identifying, solving and preventing drug-related problems in order to improve health and quality of life through pharmaceutical care in the community are therefore also important. Equally, the optimal utilization of pharmacist and clinician know – how will bring tremendous benefits to the patients from the viewpoint of social pharmacy in the promotion of safe and efficient use of drugs.

The use of combination drug therapy for cardiovascular (CV) disease risk reduction is the established approach to multiple risk factor reduction. The spectrum of rational combination products in cardiovascular disease prevention and treatment alone is extremely broad. Development of fixed-dose combination drug products requires information beyond that needed for approval of single active ingredient products. Establishing the rationale and target populations for novel combinations, as well as the contributions of the component

drugs to the claimed effects, and characterizing the pharmacokinetics, the pharmacodynamics, and clinical safety and efficacy of the combination are all necessary to support approval.

Nowadays, Telmisartan and Chlorthalidone are used for treatment of hypertension. The combined dosage form used for the treatment of prolonging hypertension and improve the quality of life. There are several methods were reported for the estimation of Telmisartan and Chlorthalidone individually or combination with other drugs. But there are no methods were reported for estimation of these drugs in combined dosage form without prior separation.

The non- availability of any UV-spectroscopy, HPLC and HPTLC until now for the simultaneous analysis of the combination made it a worth-while objective to pursue the present work.

Hence the present work was aimed to develop simple, precise and accurate methods for the estimation of Telmisartan and Chlorthalidone in bulk and in combined pharmaceutical dosage form and to validate the developed methods by UV-Spectrophotometry, HPLC and HPTLC.

3.2 PLAN OF WORK

3.2.1 Survey on Literature

A complete literature survey was made on the drugs of Telmisartan and Chlorthalidone for the various physicochemical properties such as solubility, melting point, storage conditions, etc. This survey gives some basic information of the drugs and the reported analytical methods of these drugs which help in the process of analytical method development.

3.2.2 Procurement of Raw Material and Formulation

The standard raw materials drugs of Telmisartan and Chlorthalidone were obtained as the gift samples from ERIS Pharmaceuticals, Gujarat, India. Eritel-CH-40, containing Telmisartan 40 mg and Chlorthalidone 12.5 mg was procured from local pharmacy, Melmaruvathur.

3.2.3 Method Development

The solubility of individual drugs was checked and from the list of solvents the common and stable solvent for the two drugs was selected. The solvent selected for both UV spectroscopy, HPLC and HPTLC must be cheap and readily available.

The various steps involved in the method development as follows,

UV Spectroscopy

- Selection of appropriate analytical wavelength and selection of suitable method
- Determination of working concentration range
- Analysis of synthetic mixture
- Simultaneous analysis of the formulation by using the developed method

HPLC Method

- Optimization of chromatographic conditions
- Analysis of formulation
- System suitability testing

HPTLC Method

- Optimization of chromatographic conditions
- Analysis of formulation
- System suitability testing

3.2.4 Validation

The method to be developed should be validated as per ICH and USFDA guidelines. The various parameters of validation are Linearity, Range, Precision, Accuracy, LOD, LOQ and Ruggedness.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Drug substance

The standard raw materials drugs of Telmisartan and Chlorthalidone were obtained as the gift samples from ERIS Pharmaceuticals, Gujarat, India.

4.1.2 Formulation Used

Eritel-CH-40, containing Telmisartan 40 mg and Chlorthalidone 12.5 mg was procured from local pharmacy, Melmaruvathur.

4.1.3 Chemicals and Solvents Used

Methanol (HPLC grade), Acetonitrile (HPLC grade), Water for HPLC, Toluene (AR grade), 2- Propanol (AR grade), Ammonia (AR grade) and Sodium hydroxide (AR grade) were purchased from Qualigens India Pvt. Limited, Mumbai and Loba Chemie India Limited, Mumbai. Distilled water was obtained from Double distillation unit in our laboratory.

4.1.4 Instruments Used

Different instruments used to carry out the work are,

- Shimadzu AUX- 220 Digital balance.
- Shimadzu- 1700 Double Beam UV-Visible Spectrophotometer with a pair of 10 mm matched quartz cells.
- ELICO SL-210 Double Beam UV- Visible Spectrophotometer with pair of 10 mm matched quartz cells.
- Soltec - Sonica Ultrsonic Cleaner - Model 2200 MH.
- Remi Centrifuge Apparatus
- Cyberlab Micropipette.
- HPLC
    - Shimadzu SPD – 20A UV – Visible detector.
    - Shimadzu LC – 20 AD solvent delivery module

4.1.5 Specifications of Instruments

4.1.5.1. Double beam UV-Visible Spectrophotometer (Shimadzu Instruction Manual)

Model: Shimadzu, UV-1700; Cuvetts: 1 cm matched quartz cells.

SPECIFICATIONS	
Light source	20 W halogen lamp, Deuterium lamp. Light source position automatic adjustment mechanism
Monochromators	Aberration- correcting concave blazed holographic grating
Detector	Silicon photodiode
Stray Light	0.04% or less (220 nm: NaI 10 g/lt) 0.04% or less (340 nm: $NaNO_2$ 50 g/lt)
Measurement wavelength range	190 ~ 1100 nm
Spectral band width	1nm or less (190 to 900 nm)
Wavelength accuracy	±0.5 nm on board automatic wavelength calibration mechanism
Recording range	Absorbance: -3.99~3.99 Abs Transmittance: -3.99~3.99 Abs
Photometric accuracy	±0.004 Abs (at 1.0 Abs), ±0.002 Abs (at 0.5 Abs)
Operating temperature/ humidity	Temperature range: 15 to 35° C Humidity range: 35 to 80% (15 to below 30° C) 35 to 70% (30 to below 35° C)

4.1.5.2. Shimadzu AUX- 220 digital balance (Shimadzu instruction manual)

SPECIFICATIONS	
Weighing capacity	200 gms
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operating temperature range	5 to 40° C

4.1.5.3. Elico - SL 210 Double Beams UV- Visible Spectrophotometer

(Elico Instruction Manual)

SPECIFICATIONS	
Light source	Tungsten halogen lamp (W), Deuterium lamp (D_2). Light source positions automatic adjustment mechanism
Monochromators	Concave holographic grating with 1200 lines/mm
Detector	Photodiode
Stray light	<0,05%T at 220 nm with NaI 10 g/lt
Measurement Wavelength range	190 to 1100 nm
Spectral Band Width	1.8 nm
Wavelength Accuracy	±0.5 nm
Spectral repeatability	±0.2 nm
Spectral readability	0.1 nm
Recording range	±3.0000 Abs
Photometric accuracy	±0.005 Abs (at 1.0 Abs), ± 0.010 Abs (at 1.5 abs)
Operating temperature/ Humidity	Temperature range: 15 to 35º C Humidity range: 35 to 80% (15 to below 30º C) 35 to 70% (30 to below 35º C)

C) Shimadzu HPLC(Shimadzu Instruction Manual)

Detector Specifications	
Light source	Deuterium arc lamp
Wavelength range	190 to 700 nm
Spectral Band Width	5 nm
Wavelength Accuracy	± 1 nm
Cell path length	10 nm
Cell volume	20 μl
Operating temperature range	4 to 40°C (39 to 104°F)

Recording range	0.0001 to 4.000 AUFS
Operating Temperature/ Humidity	4 to35°C/ 75 %
Pump Specifications	
Pump type	Double reciprocating plunger pump
Pumping methods	Constant flow delivery and constant pressure delivery
Suction filter	45 μm
Line filter	5 μm mesh
Operating temperature	4 to 40°C

4.1.5.4. CAMAG Automatic TLC Sampler 4 (ATS4)

APPLICATOR SPECIFICATIONS	
Spray gas	Nitrogen
Sample solvent type	Methanol
Spray gas temperature	Unheated
Syringe size	25 μl
Number of tracks	15
Application type	Band
Band length	8 mm

4.1.5.5. CAMAG TLC Scanner 3

SPECIFICATIONS	
Lamp	D_2 and W lamp
Scanning Speed	20 mm/ sec
Data Resolution	100 μm/ step
Measurement Type	Remission
Measurement Mode	Absorption

Optical Filter	Second Order
Detector Mode	Automatic

4.2 METHODS

An effort was made to develop and validate simple, accurate and precise methods for the simultaneous analysis of Telmisartan and Chlorthalidone in bulk and in pharmaceutical dosage form by

1. UV Spectrophotometry

   First order derivative spectrophotometry

2. HPLC method

3. HPTLC method.

4.2.1 UV Spectrosphotometry

4.2.1.1 First Order Derivative Spectrophotometry

Derivative spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, zero order or D° spectrum. The first derivative (D_1) spectrum is a plot of the ratio of change of absorbance with wavelength against wavelength.

4.2.1.1.2 Selection of Solvent

The solubility of drugs was determined in a variety of polar and non-polar solvents as per IP specification. The common and stable solvent was found to be 0.l M Sodium Hydroxide and dilutions were made with same 0.1 M Sodium Hydroxide for the analysis of Telmisartan and Chlorthalidone for the proposed method.

4.2.1.1.3 Preparation of Standard Stock Solutions

50 mg and 40 mg of Telmisartan and Chlorthalidone were weighed accurately and transferred in to 100 ml volumetric flasks. Dissolved with 0.1 M Sodium Hydroxide and made up to the volume with same 0.1 M Sodium Hydroxide. The stock solution contains 500 µg/ ml and 400 µg/ ml of Telmisartan and Chlorthalidone Respectively.

4.2.1.1.4 Selection of Wavelength

10 µg/ ml concentration solutions of Telmisartan and Chlorthalidone were prepared from the stock solution and the spectra were recorded between 200 nm - 400 nm by using

0.1 M sodium Hydroxide as blank. The zero order spectra were derivitized to first order and the spectra were overlain. From the overlain spectra, the wavelength selected for the analysis was 251 nm and 311 nm for Chlorthalidone and Telmisartan respectively.

4.2.1.1.5 Stability Studies

The stability of Telmisartan and Chlorthalidone were checked using 10 µg/ ml solutions at the selected wavelengths in different time intervals. It was found that Telmisartan was stable more than 5 hours, and Chlorthalidone was stable up to 4 hours 30 minutes.

4.2.1.1.6 Linearity and Calibration

From the stock solution Telmisartan, 1 – 8 ml was transferred in to a series of 100 ml volumetric flasks and made up to the volume with 0.1M Sodium hydroxide to get concentrations of 5 to 40 µg/ ml of Telmisartan. From the stock solution of Chlorthalidone, 0.5 – 6 ml were transferred in to series of 100 ml volumetric flask individually and made up to the volume with 0.1 M Sodium hydroxide to get the concentrations of 2– 24 µg/ ml of Chlorthalidone. The absorbance of the solutions was measured and the calibration graph was constructed

4.2.1.1.7 Synthetic Mixture

1 to 7 ml and 0.5 to 6 ml were pipetted out from Telmisartan and Chlorthalidone stock solutions, respectively into a series of six 100 ml volumetric flasks and made up to 100 ml with 0.1M sodium Hydroxide and to get a mixture of Telmisartan and Chlorthalidone in the concentration range of 5 to 35 µg/ ml and 2 to 24 µg/ ml, respectively. The absorbances of the prepared synthetic mixtures were measured at the selected wavelengths. The amount of drugs in the prepared synthetic mixture was calculated.

4.2.1.1.8 Quantification of Formulation

Twenty Tablets of (Eritel-CH 40, containing Telmisartan 40 mg and Chlorthalidone 12.5mg) were accurately weighed and the average weight was calculated. The tablets were crushed and made in to a fine powder. The tablet powder equivalent to 80 mg of Telmisartan was accurately weighed and transferred into 100 ml volumetric flasks. Dissolved with 0.1 M sodium hydroxide and shaken upto15 minutes. The volume was made up to 100 ml with 0.1 M sodium hydroxide. The solution was filtered through Whatmann filter paper No.41. From the stock solution, 2 ml was pipetted out and further diluted to 100 ml with 0.1 M Sodium hydroxide to get the theoretical concentrations of 16 µg/ ml and 5 µg/ ml of

Telmisartan and Chlorthalidone, respectively. The absorbances of solutions were measured at 251 nm and 311 nm. The procedure was repeated for six times. The amounts of these drugs were calculated.

4.2.1.1.9 Recovery Studies

4.2.1.1.9.1 Preparation of Raw Material Standard Stock Solutions

320 mg of Telmisartan raw material was weighed accurately in to a 10 ml volumetric flask, dissolved with 0.1M Sodium hydroxide and made up to 10 ml with 0.1 M Sodium hydroxide to get a concentration of 32 mg/ ml of Telmisartan. Similarly 100 mg of Chlorthalidone raw material were weighed accurately and transferred into 10 ml volumetric flasks, dissolved with 0.1M Sodium hydroxide and made up to 10 ml with 0.1 M Sodium hydroxide to get concentrations of 10 mg/ ml of Chlorthalidone.

4.2.1.1.9.2 Recovery Procedure

The recovery study was done by adding a specified quantity of raw material was added in to the pre analysed formulation. The tablet powder equivalent to 80 mg of Telmisartan was weighed accurately and added 2 ml, 2.5 ml, and 3.0 ml of raw standard stock solutions of Telmisartan and Chlorthalidone into three separate 100 ml volumetric flasks. Dissolved in 0.1M sodium hydroxide and shaken up to 15 minutes and made up to 100 ml with 0.1M sodium hydroxide. The solutions were filtered through Whatmann filter paper No.41. From these solutions, 2 ml was transferred into six 100 ml volumetric flasks separately and made up to the volume with 0.1M sodium hydroxide. The absorbances of the solutions were measured at 251 nm and 311 nm. The amount of drug recovered was calculated. The procedure was repeated three times for each concentration.

4.2.1.1.10 Validation of Developed Methods

4.2.1.1.10.1. Linearity

A calibration curve was plotted with concentration versus the absorbance value. The linearity was checked for Telmisartan in the concentration range of 5 to 40 µg/ ml, Chlorthalidone in the concentration range of 2 to 24µg/ ml. The drugs were found to be linear in the specified concentration ranges.

4.2.1.1.10.2. Precision

The repeatability of the method was confirmed by repeated analysis of the formulation for six times with the same concentration. The amount of drug present in the formulation was calculated. The percentage RSD value was calculated. Confidence Interval (95%) was also calculated.

The intermediate precision was confirmed by intraday and interday analysis i.e. the analysis was performed three times on the same day and on three successive days. The amount of drug present in the formulation was calculated. The percentage RSD values were calculated.

4.2.1.1.10.3. Ruggedness

Ruggedness of the method was confirmed by the analysis of formulation in different analyst. The amount of drug was calculated and the percentage RSD values were also calculated.

4.2.1.1.10.4. Accuracy

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of the standard drug solutions were added and the amount of drugs recovered were calculated. The percentages RSD values were calculated. Confidence Interval (95%) was also calculated.

4.2.1.1.10.5. LOD and LOQ

The linearity study was carried out for six times. The LOD and LOQ were calculated based up on the calibration curve method. The LOD and LOQ were calculated using the average of slope and standard deviation of intercept.

4.2.2. REVERSE PHASE – HPLC METHOD

In RP – HPLC, the retention of a compound is determined by its polarity, pKa value, molecular weight, experimental conditions, mobile phase, column and temperature. The column (typically octyl (C_8) and octadecyl (C_{18}) bonded phase) is less polar than the water – organic phase, usually an almost or entirely mobile phase. Sample molecules partition between the polar mobile phase and non – polar C_8 and C_{18} stationary phase and more hydrophobic (non - polar) compounds are retained more strongly. Polar compounds are less strongly held and elute from the column first and vice versa. Usually the lower the polarity of the mobile phase, higher in its elution strength. RP – HPLC columns are efficient, stable and reproducible because of the solvents used. Generally gradient and isocratic elution techniques used for elution and an isocratic elution technique employed for resolution of compounds in present study.

4.2.2.1. METHOD DEVELOPMENT AND OPTIMIZATION OF CROMATOGRAPHIC CONDITIONS

4.2.2.1.1. Selection of mobile phase and λ_{max}

Solutions of Telmisartan and Chlorthalidone (10 μg/ ml) were prepared in the mobile phase and scanned in the UV region of 200 – 400 nm and recorded the spectrums. It was found that these two drugs have marked absorbance at 229 nm and can be effectively used for estimation of these drugs without interference. Therefore 229 nm was selected as detection wavelength for the estimation of Telmisartan and Chlorthalidone by RP – HPLC with an isocratic elution technique.

4.2.2.1.2. Stability of sample solutions

Solutions of Telmisartan and Chlorthalidone (10 μg/ ml) were prepared and absorbance was checked for their stability at 229 nm and it was found that these two drugs were stable for 3 hours.

4.2.2.2. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

4.2.2.2.1. Initial separation conditions

The following chromatographic conditions were preset initially to get better resolution of Telmisartan and Chlorthalidone.

Mode of operation	-	Isocratic
Stationary phase	-	C_{18} column (150 mm × 4.6 mm i.d. 5μ)
Mobile phase	-	Acetonitrile: Methanol
Proportion of mobile phase	-	50: 50% v/v
Detection wavelength	-	229 nm
Flow rate	-	1ml/ min
Temperature	-	Ambient
Sample load	-	20 μl
Method	-	External Standard Calibration method.

The mobile phase was primarily allowed to run for 30 minutes to recorded a steady baseline. Mixture of solutions of Telmisartan and Chlorthalidone were injected and the respective chromatogram was recorded. It was found that both Telmisartan and

Chlorthalidone were eluted with broad peak. For this reason, different ratios of mobile phase with different solvents were tried to obtain good chromatogram with acceptable system suitability parameters.

S.No	Mobile phase	Observation
1	Acetonitrile : methanol (50: 50% v/v)	Telmisartan and Chlorthalidone were eluted with broad peak
2	Acetonitrile: Methanol (70: 30% v/v)	Telmisartan and Chlorthalidone were eluted with broad peak and resolution was less.
3	Acetonitrile: Water (70: 30% v/v)	Resolution between Telmisartan and Chlorthalidone was not obtained.
4	Acetonitrile: Water (50:50% v/v)	Peaks were eluted with better resolution but peak shape was not good for Chlorthalidone
4	Acetonitrile: 10Mm Ammonium Acetate Buffer (pH3) adjust with triethylamine. 50:50% v/v	Telmisartan and Chlorthalidone were eluted as broad peak with tailing.
5	Acetonitrile: 10Mm Ammonium Acetate (pH 7) adjust with tri ethylamine 50: 50 %v/v	Telmisartan and Chlorthalidone were eluted as broad peak with tailing.
6	Acetonitrile: 10Mm Potassium di hydrogen orthophosphate (pH3) adjust with orthophosphoric acid 50:50%v/v	Telmisartan and Chlorthalidone peaks were merged
7	Acetonitrile: Methanol: 10Mm potassium dihydrogen ortho phosphate buffer 50:20:30 v/v/v	Telmisartan and Chlorthalidone were eluted with good separation but tailing on both peaks
8	Acetonitrile: Water (50: 50% v/v) with 0.1 ml of 0.1% Triethylamine	The peaks were found to be good with better resolution

With the above information's, Acetonitrile: water with 0.1ml of 0.1% triethylamine was selected as mobile phase and further optimization was done with this mobile phase.

4.2.2.2.2. Effect of ratio of mobile phase

With the above selected mobile phase, different composition was tried with the different ratios of Acetonitrile and Water. The ratios tried were 50:50, 70:30, 30:70 and 60:40 % v/v. At 50:50 ratios the peaks obtained were very sharp with better resolution. Hence this ratio was selected for further analysis.

4.2.2.3. Optimized chromatographic conditions

The following optimized chromatographic conditions were employed for analysis of Telmisartan and Chlorthalidone by isocratic RP – HPLC method.

Mode of operation	-	Isocratic
Stationary phase	-	C_{18} column (150 mm × 4.6 mm i.d. 5μ)
Mobile phase	-	Acetonitrile : Water with 0.1 ml of 0.1 % Triethylamine
Proportion of mobile phase	-	50:50% v/ v
Detection wavelength	-	229 nm
Flow rate	-	1ml/ min
Temperature	-	Ambient
Sample load	-	20 μl
Operating pressure	-	140kgf
Method	-	External Standard Calibration method.

4.2.2.3.1. Preparation of standard Telmisartan solution

25 mg of Telmisartan was weighed accurately and transferred into 25 ml volumetric flask and dissolved in methanol, after dissolution the volume was made up to the mark with methanol (1000μg/ ml). Further dilution was made by pipetting 2.5 ml of mother liquor into 25 ml with mobile phase to acquire 100 μg/ ml solution.

4.2.2.3.2. Preparation of standard Chlorthalidone solution

25 mg of Chlorthalidone was weighed accurately and transferred into 25 ml volumetric flask and dissolved in methanol, after dissolution the volume was made up to the mark with methanol (1000 μg/ ml). Further dilution was made by pipetting 2 ml of mother liquor into 25 ml with mobile phase to acquire 80μg/ ml solution.

4.2.2.3.3. Preparation of Calibration graph

In this progression, the aliquots of stock solution of Telmisartan and Chlorthalidone (0.5 – 2.5 ml) and (0.5 – 2 ml) individually were transferred into a series of 10 ml volumetric

flasks and made up to the mark with mobile phase. The solutions containing the concentrations of 5- 25 μg/ ml of Telmisartan and 2 – 16 μg/ ml of Chlorthalidone. All the solutions were injected and the chromatograms were recorded at 229 nm. The above concentration range was found to be linear and obeys Beer's law. The procedure was repeated for three times. The peak areas were plotted against concentration and the calibration curve was constructed.

4.2.2.3.4. Estimation of Telmisartan and Chlorthalidone in tablet formulation

Estimation of Telmisartan and Chlorthalidone in tablet formulation by RP – HPLC was carried out using optimized chromatographic conditions. Ten tablets of formulation (Eritel-CH 40 containing 40 mg of Telmisartan, 12.5mg of Chlorthalidone) were weighed accurately. The average weight of tablets was found and powdered. The tablet powder equivalent to 80 mg of Telmisartan was weighed and transferred into 100 ml volumetric flask and added about 80 ml of methanol to dissolve the substance and made up to the volume with the same (800 μg/ ml). The solution was sonicated for 15 minutes, centrifuged at 200 rpm for 15 minutes and filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were made by diluting 1 ml into 10 ml with mobile phase to obtain 80 μg/ ml solution. This solution was used for further analysis.

4.2.2.3.5. Assay Procedure

1ml of test solution was transferred into six 10 ml volumetric flasks individually and made up to the mark with mobile phase to obtain 8 μg/ ml. A steady base line was recorded with optimized chromatographic conditions. After the stabilization of base line for 30 minutes, the test solutions were injected and recorded the chromatograms. The concentration of each test solution was determined by using slope and intercept values from the calibration graph.

4.2.2.3.6. Recovery Experiments

a) Preparation of pre analysed formulation stock solution

The powdered tablet equivalent to 80 mg of Telmisartan was weighed and transferred into 100 ml standard flask and added about 80 ml of methanol to dissolve the substance and made up to the volume with methanol (800 μg/ ml). The solution was sonicated for 15 minutes, centrifuged at 200 rpm for 15 minutes and filtered through Whatmann filter paper No. 41. From the clear solution, further 1ml transferred into 10 ml volumetric flask and diluted 10 ml with mobile phase to obtain 80 μg/ ml solution.

4.2.2.3.7. Procedure

Formulation equivalent to 80 mg of Telmisartan and added 64 mg, 80 mg and 96 mg raw material of Telmisartan and 20 mg, 25 mg and 30 mg raw material of Chlorthalidone into 100 ml volumetric flasks separately and made up to the mark with methanol. Further procedure was followed as per the analysis of formulation. The solutions were injected and the chromatograms were recorded at 229 nm. The quantity of drug recovered was calculated by using slope and intercept values from the calibration graph.

4.2.2.3.8. System suitability studies

The system suitability studies conceded as per ICH guidelines and USP. The parameters like capacity factor, tailing factor, asymmetry factor, number of theoretical plates and resolution were calculated.

4.2.2.3.9. Validation of Developed Method

4.2.2.3.9.1. Linearity

A calibration curve was plotted with concentration versus the peak area. The linearity range was checked for in the concentration range of 100 – 800 ng/ µl and 50 – 400 ng/ µl, ofTelmisartan and Chlorthalidone respectively. The drugs were found to be linear in the specified concentration ranges.

4.2.2.3.9.2 Precision

The repeatability of the method was checked by repeated analysis of the formulation for six times with the same concentration. The amount of drug present in the formulation was calculated. The percentage RSD value was calculated. Confidence Interval (95%) was also calculated.

The intermediate precisions were confirmed by the intraday and inter day analysis i.e. the analysis was performed three times on the same day and on three successive days. The amount of drug present in the formulation was calculated. The percentage RSD values were calculated.

4.2.2.3.9.3. Accuracy

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of the standard drug solutions were added and the amount of drug recovered was calculated. The percentages RSD values were calculated.

4.2.2.3.9.4. LOD and LOQ

The linearity study was carried out for three times. The LOD and LOQ were calculated based up on the calibration curve method. The LOD and LOQ were calculated using the average of slope and standard deviation of intercept.

4.2.3. HPTLC

In HPTLC, the separation of the components of a mixture is based on the principle of adsorption. The HPTLC differ from the TLC in the size of silica gel used as the stationary phase and automated sampling application and detection. In the present study a twin through chamber and silica 60 F 254 were used.

4.2.3.1. Selection of Stationary Phase

The resolution of Telmisartan and Chlorthalidone was achieved using TLC plate made up of silica gel G60 F 254 coated on an Aluminium support (E.Merck). The size of the silica gel particle was 2 μ and thickness of sorbent layer was 0.2 mm. The plates were supplied in 20 × 10 cm size which was cut in to appropriate sizes for method development.

4.2.3.2. Selection of Mobile Phase

The mobile phase system was chosen based on the solubility and polarity of two drugs. The solution of drugs was prepared in methanol and used for spotting. Methanol gets vaporized soon after application on to the plate under nitrogen stream. After trying different mobile phase system an ideal system was chosen based on the resolution between compounds. The fixed mobile phase system for the separation of two drugs consisted of (mobile phase) with an appropriate Rf values. The drugs were scanned at 224 nm after the development.

The velocity of mobile phase in HPTLC is affected by the nature of the stationary phase (porosity, packing, particle size, etc), as well as mobile phase properties (viscosity, surface tension, vapour pressure of solvents, etc). Generally the velocity of mobile phase decreases during chromatographic development due to higher resistance of stationary phase densely packed with fine particles.

Various mobile phase tried were

Mobile Phase	**Ratio**
0.5 M NaCl: Methanol: Acetonitrile: Glacial acetic acid	(10: 4: 6: 0.01% v/v/v/v/v)
Ethyl Acetate: 1,4- dioxane: Methanol: 25% ammonia	(15: 15: 3:1.5 % v/v/v/v/v)
Ethyl acetate: Methanol: 25% ammonia Glacial Acetic acid	7.5: 1.5: 2.0: 1.0: 0.2 % v/v/v/v/v)
Toluene: Benzene: Methanol	(5: 3: 2 % v/v/v)

Benzene: Toluene: Diethylamine	(5: 3: 2 % v/v/v)
Benzene: Toluene: n-butanol	(5: 3: 2% v/v/v)
THF: dichloromethane: Methanol: 25% ammonia	(6: 2: 1: 0.4% v/v/v/v/v)
THF: dichloromethane: Methanol: 25% ammonia	(6: 2: 1: 0.2% v/v/v/v/v)
Toluene: 2-Propanol:25% ammonia	(5.5: 4.5: 0.02% v/v)
Toluene: 2-Propanol:25% ammonia	(6.5: 3.5: 0.02% v/v)
Toluene: 2-Propanol:25% ammonia	(4.5: 5.5: 0.02% v/v)
Toluene: 2-Propanol:25% ammonia	(3.5: 6.5: 0.02% v/v)

From the above list of mobile phase the mobile phase Toluene: 2- Propanol: 25% ammonia (6.5: 3.5: 0.02% v/v) was found to be an ideal mobile phase with good resolution between the spots.

4.2.3.3. Optimization of Variants in TLC

The composition of mobile phase, chamber saturation (equilibration time), plate equilibration time, the distance of solvent development and bandwidth of the spot are a few variants affect Rf values of drugs.

4.2.3.4. Chamber Saturation (Equilibration time)

Chamber saturation is done so that equilibration is established eventually between the components of developing solvents and their vapour phase and the formation of secondary solvent fronts could be avoided

Hence in the current study chamber saturation was taken in to consideration to achieve reproducible Rf values and peak area. The mobile phase was placed on one side of twin through chamber and shaken well. Different saturation times were maintained for different mobile phase. The chamber saturation time for Toluene: 2- Propanol: 25% ammonia (6.5: 3.5: 0.02% v/v) was 30 minutes.

4.2.3.5. Selection of Detection Wavelength

By comparing the spectral characters of Telmisartan and Chlorthalidone the detection wavelength was selected for the method 229 nm was selected for as the detection wavelength with reference to the spectral conformation graph.

4.2.3.5.1. Optimized Chromatographic Conditions

After conforming with the mobile phase and detection wavelength, the optimized conditions for the method was as follows

Stationary Phase	-	Silica Gel 60 F 254 HPTLC Plates
Mobile Phase	-	Toluene: 2- Propanol: 25% ammonia
Mobile Phase ratio	-	(6.5: 3.5: 0.02% v/v)
Detection	-	CAMAG TLC scanner 3, at 229 nm
Temperature	-	Room Temperature
Chamber	-	Twin through Chamber
Development Mode	-	Ascending Mode

4.2.3.5.2. Preparation of Standard Stock Solution

100 mg of Telmisartan raw material was accurately weighed into 50 ml volumetric flask and 100 mg of Chlorthalidone raw material was accurately weighed into 100 ml of volumetric flask, dissolved in methanol and made up to the volume with methanol. The solution contains 2 mg/ ml of Telmisartan and 1 mg/ml of Chlorthalidone.

4.2.3.5.3. Linearity and Calibration Curve

0.5- 4.0 ml of the standard stock solutions of Telmisartan and Chlorthalidone were pipetted out into a serious of 10 ml volumetric flasks and made up to the volume with methanol. 1µl solutions were spotted on TLC plates and to get the concentration range of 100 – 800 ng/ µl, and 50 – 400 ng/ µl of Telmisartan and Chlorthalidone, respectively.

4.2.3.5.4. Quantification of Formulation

Twenty Tablets were weighed accurately and the average weight of each tablet was determined. The tablet powder equivalent to 32 mg of Telmisartan into a series of six 100 ml volumetric flasks. Dissolved in methanol and sonicated for 15 minutes. The volume was made up to 100 ml with methanol and was centrifuged for 15 minutes at 2000 rpm. The supernatant solution was filtered through whatmann filter paper No.41. The solution contains 320 ng/µl, 100 ng/ µl, of Telmisartan and Chlorthalidone, respectively. 1 µl spots were placed on the plates and the chromatogram was developed in the twin through chamber. From the peak area and amount of drugs were calculated. The procedure was repeated for six times.

4.2.3.5.5. Recovery Studies

4.3.3.5.5.1. Procedure

To the tablet formulation equivalent to 32 mg of Telmisartan, added 25.6 mg, 32 mg and 38.4 mg of raw material of Telmisartan and 8 mg, 10 mg and 12 mg of raw material of Chlorthalidone into 100 ml volumetric flasks separately and made up to the mark with

methanol. Further procedure was followed as per the analysis of formulation. The solutions were injected and the chromatograms were recorded at 229 nm. The quantity of drug recovered was calculated by using slope and intercept values from the calibration graph.

4.2.3.6 Validation of Developed Method

4.2.3.6.1. Linearity

A calibration curve was plotted with concentration versus the peak area. The linearity range was checked for in the concentration range of 100 – 800 ng/ µl and 50 – 400 ng/ µl, of Telmisartan and Chlorthalidone respectively. The drugs were found to be linear in the specified concentration ranges.

4.2.3.6.2. Precision

The repeatability of the method was checked by repeated analysis of the formulation for six times with the same concentration. The amount of drug present in the formulation was calculated. The percentage RSD value was calculated.

The intermediate precisions were confirmed by the intraday and inter day analysis i.e. the analysis was performed three times on the same day and on three successive days. The amount of drug present in the formulation was calculated. The percentage RSD values were calculated.

4.2.3.6.3. Accuracy

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of the standard drug solutions were added and the amount of drug recovered was calculated. The percentages RSD values were calculated.

4.2.3.6.4. LOD and LOQ

The linearity study was carried out for three times. The LOD and LOQ were calculated based up on the calibration curve method. The LOD and LOQ were calculated using the average of slope and standard deviation of intercept.

RESULT AND DISCUSSION

5. RESULTS AND DISSCUSION

Simultaneous estimation of two drugs in a formulation has more advantages such as accurate, less use of reagent and less time requirement for the estimation rather than individual estimation of two drugs. The simple, precise and accurate analytical techniques were developed for the simultaneous estimation of Telmisartan and Chlorthalidonein bulk and in pharmaceutical dosage form. The methods include

1. UV Spectroscopy

- First Order Derivative Spectrophotometry

2. HPLC Method

3. HPTLC Method

5.1. UV Spectroscopy

5.1.1 First Order Derivative Spectrophotometry

A simple, precise and accurate first order derivative spectrophotometric method was developed and validated for the simultaneous estimation of Telmisartan and Chlorthalidone in bulk and in combined dosage form. The drugs were identified by the melting point and IR spectrum, the IR spectrum of Telmisartan and Chlorthalidonewere given in figures 1and 2, respectively. The solubility of drugs in various polar and non polar solvents checked as per IP specifications. All the drugs were exhibited different solubility characters. From the solubility data, the common solvents were found to be acetone, acetonitrile, dimethyl formamide, di ethylamine, ethanol, methanol, n-butanol, and 0.1M sodium hydroxide. The solvents such as acetonitrile, acetone, dimethyl formamide,di ethylamine and n-butanol, were not selected because the cut off wavelength of these solutions were above 240 nm and the solvent interference should be expected during the analysis.Hence, 0.1M sodium hydroxide was selected as the solvent for the analysis of Telmisartan and Chlorthalidone. The solubility profile of Telmisartan and Chlorthalidone were given in tables 1 and 2,respectively.

10 µg/ ml concentration solutions of Telmisartan and Chlorthalidone were prepared and the spectra were recorded. The overlain zero order spectra are shown in figure 3. By observing the spectral characters of Telmisartan and Chlorthalidone the methods used for the multi component analysis viz. simultaneous equation method, absorption correction method and absorption ratio method were not applied, because the interference were more. Hence the normal curve was derivitized to first order and overlained as shown in figure 4. From the overlain spectra 311nm and 251 nm were selected for the simultaneous estimation of

Telmisartan and Chlorthalidone, respectively. At 311 nm, Telmisartan has the absorbance where as Chlorthalidone has no absorbance. Hence this wavelength selected for the analysis of Telmisartan without the interference of Chlorthalidone. At 251 nm Chlorthalidone has the absorbance where as Telmisartan have zero crossing points.Hence this wavelength selected for the analysis of Chlorthalidone without the interference of Telmisartan.

The stability of Telmisartan and chlorthalidone were checked at the selected wavelengths using 0.1M sodium hydroxide as solvent. It was found that Telmisartan was stable for more than 5 hours and Chlorthalidone for about 4 hours 30 minutes.

Various aliquots of Telmisartan and Chlorthalidone were prepared in the concentration range of 5 - 40 µg/ ml and 2 – 24 µg/ ml, respectively. The absorbances of these solutions were measured at the selected wavelengths. The calibration curve was constructed using concentration versus absorbance. The preparation of calibration curve was repeated for six times for each drug at their selected wavelengths. The optical parameters like Molar absorptivity, Sandell's sensitivity, Correlation coefficient, Slope, Intercept, LOD and LOQ were calculated. The correlation coefficient for all the four drugs was found to be above 0.999. This indicates that all the drugs obey Beer's law in the selected concentration ranges. Hence the concentrations were found to be linear. The calibration graph for Telmisartan at 311 nm and for Chlorthalidone at 251 nm are shown in figure 5 and 6, respectively. The optical characteristics of the drugs at their selected wavelengths are shown in tables 3 and 4, respectively.

The developed method was applied for the analysis of synthetic mixture to find out the developed method was correct or not. The amount of Telmisartan and Chlorthalidone were found to be in the range of 99.80-102.07 % and 97.54–99.36%, respectively. The results are listed in table 5. The amount found was good agreement with the expected concentration. Hence it was planned to apply for the analysis of formulation.

The tablet formulation Eritel- CH 40 (containing Telmisartan- 40 mg and Chlorthalidone) was selected for the analysis. The percentage purity of the drugs in the formulation was found to be 100.33 ±0.7993 and 99.99 ± 0.4373 for Telmisartan and Chlorthalidone, respectively. The results are listed in table 6. The confidence interval (95%) for drugs was found to be in the range of, 99.0142 – 101.6458 and 99.0143 – 100.7098 for Telmisartan and Chlorthalidone, respectively. The precision of the method was confirmed by

the repeated analysis of formulation for six times. The percentage RSD values were found to be 0.7966 and 0.4374 for Telmisartan and Chlorthalidone, respectively.

Further the precision of the method was confirmed by intraday and inter day analysis. Intraday and inter day analysis of formulation was done on three times on same day and one time on three consecutive days. The percentage RSD for the intraday and inter day precision was found to be 0.9295 and 0.8424 for Telmisartan 1.2105 and 1.1785 for Chlorthalidone, respectively. The results are listed in table 7. The low percentage RSD values indicated that the precision of the method was confirmed.

The ruggedness of the method was validated by using different analysts. The percentage RSD for analyst 1 were found to be 0.7643 and 0.4279 for Telmisartan and Chlorthalidone, respectively. The result shown in table 8.

The accuracy of the method was confirmed by the recovery studies. To the pre-analyzed formulation, a known quantity of raw material was added and the percentage recovery was calculated. The percentage of raw material added was 80%, 100% and 120% for Telmisartan and Chlorthalidone Respectively. The percentage recovery was found to be in the range of 99.13- 102.72 % for Telmisartan, 99.98 – 101.52 for Chlorthalidone. The percentage RSD value was found to be 1.7823 for Telmisartan, 1.1152 for Chlorthalidone, The low percentage RSD indicated there was no interference due to excipients used in formulation. Hence, the accuracy method was conformed. The results are listed in table 9. The confidence interval (95%) for drugs was found to be in the range of 100.26 – 102.77, and 98.85-102.87 for Telmisartan and Chlorthalidone, respectively.

5.3 RP – HPLC METHOD

An effort has been made for a simple, rapid, accurate and precise method for the simultaneous estimation of Telmisartanand Chlorthalidone in pure form and in formulation by an isocratic RP – HPLC method.

The solution of 10 µg/ ml of Telmisartan and Chlorthalidone was prepared in the mobile phase of Acetonitrile: water with the ratio of 50: 50% v/v. The solutions were scanned in the UV range of 200 - 400 nm. It was found that these two drugs have marked absorbance at 229 nm and therefore 229 nm was selected as detection wavelength for estimation of two drugs by RP – HPLC method with an isocratic elution technique and it was found that these two drugs are stable for at least 3 hours. The overlinespectra is shown in figure 7.

The optimization was done by changing the composition of mobile phase, ratio and flow rate. The mobile phase consists of Acetonitrile: Methanol (50:50 % v/v) was initially tried and chromatogram was recorded. These are shown in Figure 8. The mobile phase consists of Acetonitrile : Ammonium acetate buffer pH 5 (50: 50% v/v) was tried and the chromatograms are shown in Figure 9. The mobile phase consists of Acetonitrile: Phosphate Buffer (50: 50 % v/v) pH (3.5) and 70:30% were tried and the chromatograms are shown in figures 10 and 11. Finally the mobile phase consists of Acetonitrile: Water with 0.1 ml of 0.1% triethylamine with the ratio of 50: 50% v/v, was tried. After calculating all system suitability parameters Acetonitrile: Water with 0.1 ml of 0.1% Triethylamine in the ratio of 50: 50 % v/v at flow rate of 1.0 ml/ min was selected and the optimized chromatogram is shown in figure 12. The system suitability parameters for optimized chromatogram are shown in Table 10. The retention time for Telmisartan and Chlorthalidone was found to be minutes 2.915 and 4.637 respectively and with a resolution of 9.087 which is better resolution.

The calibration was done by external standard calibration method. With the optimized chromatographic conditions, stock solutions of Telmisartan and Chlorthalidone were prepared by using methanol (for first dilution only) and mobile phase and various concentrations were prepared in the range of 5-25μg/ ml of Telmisartanand 2 - 16 μg/ ml of Chlorthalidone, respectively. 20 μl of each solution were injected individually and the chromatograms were recorded at 229 nm. The chromatograms are shown in figures 13 - 17. The calibration curve was plotted using concentration against peak area. The procedure was repeated for three times. The correlation co - efficient value was found above 0.999 for two drugs. It indicates that the concentrations of Telmisartan and chlorthalidone had good linearity. The calibration graphs are shown in figures 18 and 19. The optical characteristics of Telmisartanand Chlorthalidone are shown in Table 11.

The tablet dosage form (Eritel-CH 40) was selected for the analysis. The ostensible concentration of 8 μg/ ml of Telmisartan which is also containing 2.5 μg/ ml of Chlorthalidonein the mobile phase was prepared. 20 μl of each solution was injected and chromatograms were recorded. The percentage purity was found to be 101.63 ± 0.7025 and 101.46 ± 0.4449 for Telmisartan and Chlorthalidone, respectively. The precision of the method was confirmed by repeatability of formulation for six times and the chromatograms are shown in figures 20 – 25. The percentage RSD was found to be 0.6912 and 0.4329 for Telmisartan and Chlorthalidone, respectively. It indicates that the method has good precision. The data for the analysis of formulation is shown in Table 12.

The accuracy of the method was performed by recovery studies. To the pre analyzed formulation, a known quantity of Telmisartan and Chlorthalidone raw material solutions were added at different levels and injected the solutions. The chromatograms were recorded as shown in the Figure 26 - 28. The percentage recovery was found to be in the range between 99.45 – 101.76% for Telmisartan and 99.08 – 102.01% for Chlorthalidone. The % RSD was found to be 0.9350 for Telmisartan and 0.3580 for Chlorothalidone respectively. The low % RSD values for recovery indicated that the method was found to be accurate. The values are given in the table 13. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

5.2 HPTLC Method

An effort was made to develop a simple, precise and accurate method for the simultaneous estimation ofTelmisartan and Chlorthalidonein bulk and in pharmaceutical dosage form by HPTLC method.

The initial separation was based up on the solubility of drugs, the different mobile phase were tried to get the better resolution. The different mixtures of the mobile phase tried were Ethyl Acetate: 1,4- dioxane: Methanol: 25% ammonia, Ethyl acetate: Methanol: 25% ammonia: Glacial Acetic acid, Toluene: Benzene: Methanol, Benzene: Toluene: Diethylamine, THF: dichloromethane: Methanol: 25% ammonia. InToluene: 2-Propanol:25% ammonia, all the drugs were well separated with good resolution when compared to other mobile phases. By altering the composition of Toluene the separation was affected more. Hence the chromatograms were recorded by changing the concentration of Toluene. In the mobile phase, Toluene: 2-Propanol: 25% ammonia, in the ratio of 6.5: 3.5: 0.02% ammonia, both the drugs were eluted with better resolution. Hence this was selected as the mobile phase for the analysis of Telmisartan and Chlorthalidone. With the above selected mobile phase the UV spectra of all the drugs were recorded and overlained. From the overlain spectra, at 229 nm both the drugs showed marked absorbance. Hence this was selected as the detection wavelength. The optimized chromatogram is shown in figure 29. The Rf value for Telmisartan and Chlorthalidone were found to be 0.28 ± 0.01 and 0.65 ± 0.01 respectively.

The linearity range was fixed as 100 – 800 ng/ µl for Telmisartan and 50 – 400 ng/ µl for Chlorthalidone.shown in figures 30 – 37. The calibration graph was recorded using peak

area and concentration and these are shown in figures 38 and 39. The correlation coefficients were found to be 0.9992 and 0.9989, for Telmisartan and Chlorthalidone, respectively. The optical characteristics such as the Correlation coefficient, Slope, Intercept, LOD and LOQ and were calculated and shown in tables 14. The correlation coefficient values indicated that the selected concentration was linear.

The tablet dosage form ERITEL-CH was selected for the analysis. The chromatogram for the analysis of formulation was shown in figures 40 – 45. The percentage purity of Telmisartan and Chlorthalidone were found to be 101.66 ± 0.7839 and 101.31 ± 0.8343, respectively. The results of analysis are shown in table 15. The confidence interval(95%) for drugs was found to be in the range of 100.37 – 102.95 and 99.94 – 102.68 for Telmisartan and Chlorthalidone, respectively.Precision of the method was confirmed by repeated analysis of formulation for six times. The percentage RSD values were found to be 0.7725 and 0.8228 for Telmisartan and Chlorthalidone, respectively.

Further the precision of the method was confirmed by intraday and inter day analysis. Intraday and inter day analysis of formulation was done on three times on same day and one time on three consecutive days. The percentage RSD values for the intraday and inter day precision was found to be less than 2. The low % RSD values indicated that the intermediate precision of the method was confirmed.

The accuracy of the method was confirmed by the recovery studies. To the pre-analyzed formulation, a known quantity of raw material was added and the percentage recovery was calculated. The percentage of raw material added was 80%, 100% and 120% for two drugs. The chromatograms for the recovery analysis are shown in figures 46 – 48. The percentage recovery was found to be in the range of 98.99 - 99.23%, for Telmisartan and 101.16 - 102.38% for Chlorthalidone, The percentage RSD values were found to be 0.1201 and 0.6319 for Telmisartan and Chlorthalidone respectively. The low percentage RSD value indicates that there was no interference due to the excipients used in formulation during the analysis. The data of recovery analysis are listed in table 16.

SUMMARY

AND

CONCLUSION

6. SUMMARY AND CONCLUSION

Three simple, precise and accurate methods were developed for the simultaneous estimation of Telmisartan and Chlorthalidone in bulk and in pharmaceutical dosage form.

The methods developed were

1. UV Spectroscopy

   - First Order Derivative Spectrophotometry

2. HPLC

3. HPTLC Method

6.1. UV Spectroscopy

6.1.1 First Order Derivative Spectrophotometry

From the solubility profile, 0.1M Sodium hydroxide was chosen as the common solvent for the simultaneous analysis of Telmisartan and Chlorthalidone. The spectra of all the drugs recorded and are derivitized to first order. From the overlain first order derivative spectra 311 nm and 251nm were selected for the analysis of Telmisartan and Chlorthalidone, respectively.

Beer's law obeyed the concentration range of 5 - 40 µg/ ml, and 2-24 µg / ml, for Telmisartan and Chlorthalidone, respectively. The calibration graphs were plotted. The correlation coefficient values for the two drugs were more than 0.999. The optical parameters like the Molar absorptivity, Sandell's sensitivity, Correlation coefficient, Slope, Intercept, LOD and LOQ, and were calculated.

The developed method was applied for the analysis of synthetic mixture to find out the developed method was correct or not. The amount of Telmisartan and Chlorthalidone were found to be in the range of 99.80- 102.07% and 97.54 - 99.36%, respectively. The amount found was good agreement with the expected concentration. Hence it was planned to apply for the analysis of formulation.

ERITEL – CH 40 Tablet containing Telmisartan 40 mg and Chlorthalidone 12.5 mg and was selected for the analysis. The percentage of drugs in the formulation was found to be 100.33 ± 0.7993 and 99.90 ± 0.4373 for Telmisartan and Chlorthalidone respectively. The

confidence interval (95%) for drugs was found to be in the range of 99.01-101.64% and 99.27 - 100.70% for Telmisartan and chlorthalidone, respectively.

The precision of the method was confirmed by the repeatability studies. The percentages RSD were found to be 0.7966 and 0.4374 for Telmisartan and Chlorthalidone, respectively.

Further the precision of the method was confirmed by intraday and inter day analysis. The percentages RSD values for intraday and inter day were found to be 0.9295 and 0.8424 for Telmisartan, 1.2105 and 1.1785 for chlorthalidone.

The ruggedness of the method was confirmed by performing the analysis with the different analysts. The percentage RSD values for different analysts were found to be 0.7643 and 0.4279 for Telmisartan and chlorthalidone respectively.

The accuracy of the method was confirmed by recovery studies. To the pre analysed formulation the different amount of raw material were added and the amount of drugs recovered were calculated. The percentage recovery was found to be in the range of 99.13 - 102.72% for Telmisartan 99.98 - 101.52 % for Chlorthalidone, the percentage RSD value was found to be 1.7823 for Telmisartan 1.1152 for Chlorthalidone The confidence interval (95%) for drugs was found to be in the range of 100.26 to 102.77 and 98.25 to 101.88 for Telmisartan and Chlorthalidone, respectively.

6.2 RP – HPLC METHOD

An exertion has been made for a simple, rapid, accurate and precise method for the estimation of Telmisartan and Chlorthalidone in pure form and in formulation by an isocratic RP – HPLC method.

The optimization was done by changing the composition of mobile phase and mobile phase ratios. Finally the mobile phase consisting of Acetonitrile and Water with 0.1 ml of 0.1%Triethylamine was selected. The detection wavelength was 229 nm. The retention time ofTelmisartan and Chlorthalidone were found to be 2.915 and 4.637 respectively. The resolution between the separated components was 9.187.

The calibration was done by external standard calibration method. The drug solutions of Telmisartan and Chlorthalidone were obyed Beer's law in the concentration range of 5-25 µg/ ml and 2- 16 µg/ ml, respectively. The correlation coefficient was above 0.999 for the both the drugs.

Eritel-CH 40 tablets containing 40 mg of Telmisartan and 12.5 mg of Chlorthalidone was selected for analysis. The percentage label claim present in tablet formulation was found to be 101.63 ± 0.7025 and 101.46 ± 0.4449 for Telmisartan and Chlorthalidone, respectively.

The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 99.45 – 101.76% for Telmisartan and 99.08 - 102.01% for Chlorthalidone. The % RSD values for Telmisartan and Chlorthalidone were found to be 0.9350 and 0.3580 respectively.

6.3. HPTLC Method

The simple, precise and accurate HPTLC method was developed for the simultaneous estimation of Telmisartan and Chlorthalidone in bulk and in pharmaceutical dosage form.

The mobile phase consists of Toluene: 2-Propanol: 25% ammonia (6.5: 3.5: 0.02% v/v/v/v) was selected for the analysis. From the spectral characteristics, 229 nm was selected as detection wavelength for the analysis.

Beer's law obeyed the concentration range of 100 – 800 ng/ µl, 50 – 400 ng/ µl, for Telmisartan and Chlorthalidone, respectively. The calibration graphs were plotted. The correlation coefficient values for all the drugs were more than 0.999. The optical parameters like the Correlation coefficient, Slope, Intercept, LOD and LOQ were calculated.

Eritel-CH 40 containing Telmisartan 40 mg and Chlorthalidone 12.5 mg was selected for the analysis. The percentage of drugs in the formulation was found to be 101.66 ± 0.7725, 101.31 ± 0.8336, for Telmisartan and Chlorthalidone, respectively**.** The confidence interval (95%) for drugs was found to be in the range of 100.36 – 102.95 and 99.94 to 102.68 for Telmisartan and Chlorthalidone, respectively.

Precision of the method was confirmed by repeated analysis of formulation for six times. The percentage RSD values were found to be less than 2. The low percentage RSD value indicates that the intermediate precision of the method was conformed.

The accuracy of the method was confirmed by the recovery studies. To the pre-analyzed formulation, a known quantity of raw material was added and the percentage recovery was calculated. The percentage recovery was found to be in the range of 98.99 - 99.23%, for Telmisartan and 101.16 – 101.88 for Chlorthalidone. The percentage RSD values were found to be 0.1212 and 0.6034 for Telmisartan and Chlorthalidone,

respectively. The confidence interval (95%) for drugs was found to be in the range of 98.90 – 99.29 and 100.68 – 102.77 for Telmisartan and Chlorthalidone, respectively.

Three instrumental analytical methods were successfully developed for the simultaneous estimation of Telmisartan and Chlorthalidone in bulk and in pharmaceutical dosage form.

The three methods were found to be accurate, precise and rapid for the simultaneous estimation of these drugs. This was confirmed by low percentage RSD values. The spectrophotometric method is found to be economical when compared to the HPLC and HPTLC method. But HPLC and HPTLC is more sensitive than UV spectrophotometric method. The low percentage RSD value in the recovery studies suggests that the excipients do not interfere in the analysis of formulation and hence all the methods are accurate. HPLC and HPTLC are found to be more sensitive than other method. Because the linearity range, LOD, LOQ were less in HPLC and HPTLC method than UV spectroscopic method. Hence it is suggested that these three methods can be applied successfully for the routine quality control analysis for the simultaneous estimation of Telmisartan and Chlorthalidone in bulk and in pharmaceutical dosage form and the obtained results will be presented elsewhere.

FIGURES

Figure 1

IR SPECTRUM OF TELMISARTAN

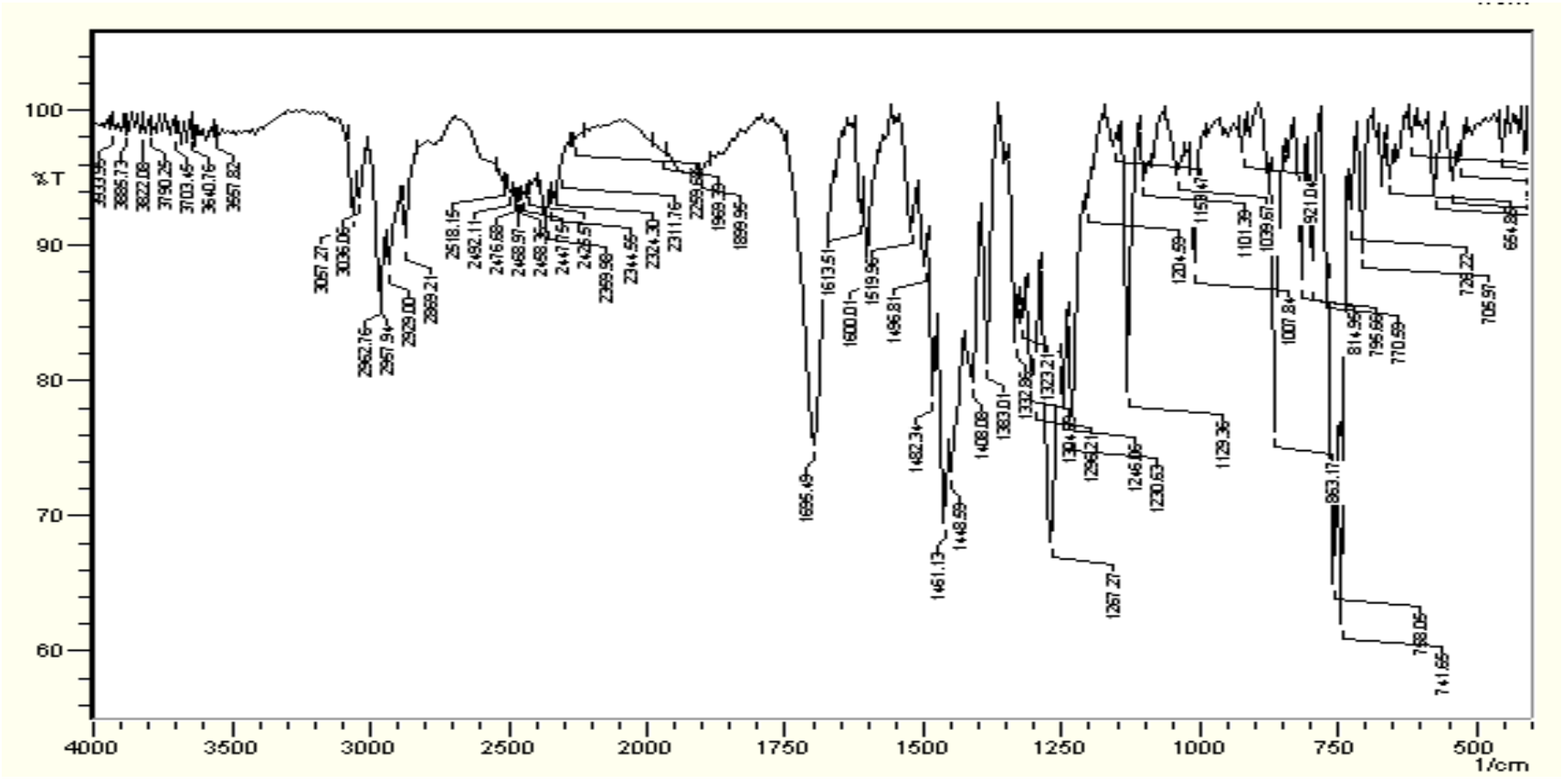


Figure 2

IR SPECTRUM OF CHLORTHALIDONE

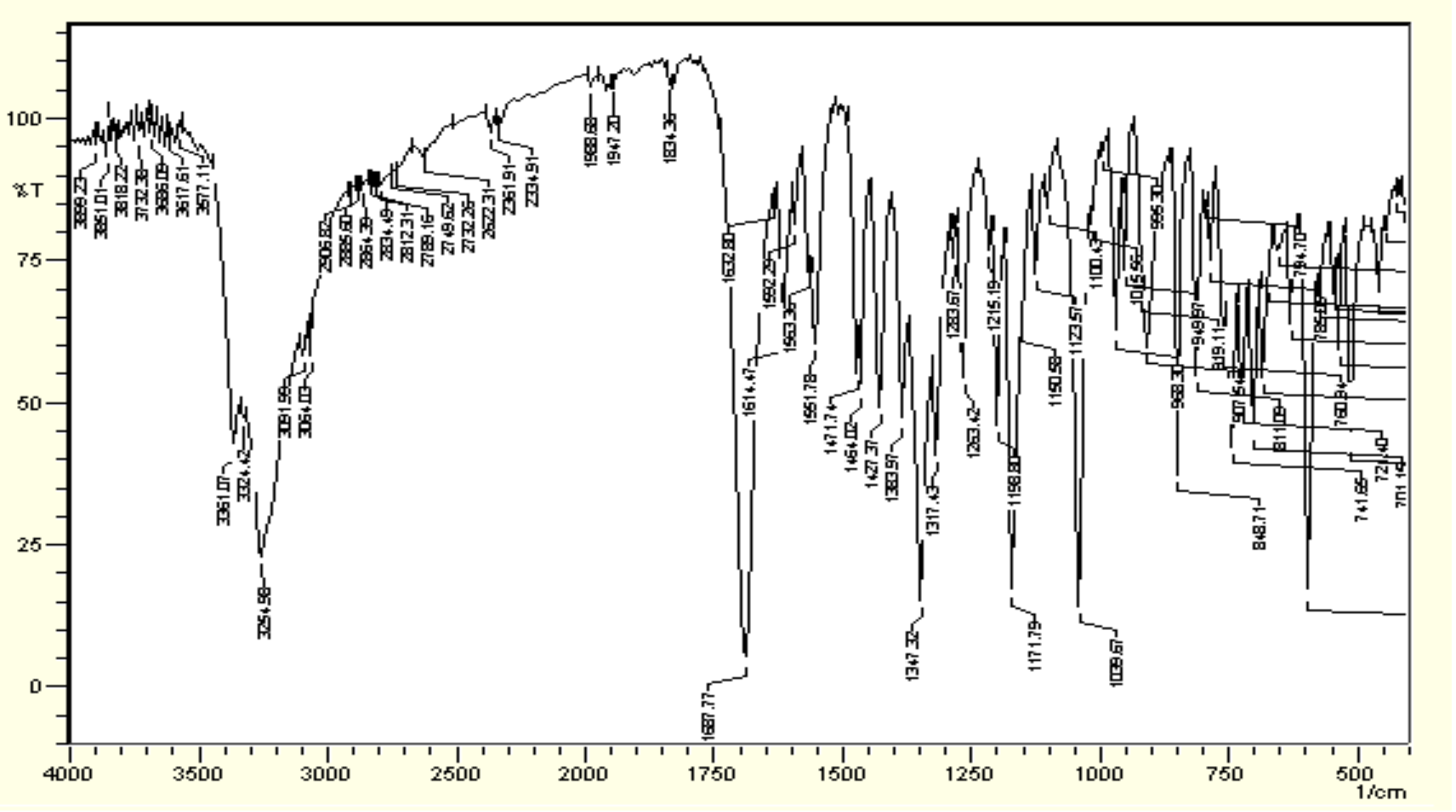


Figure 3

OVERLAIN ZERO ORDER SPECTRA OF TELMISARTAN AND CHLORTHALIDONE

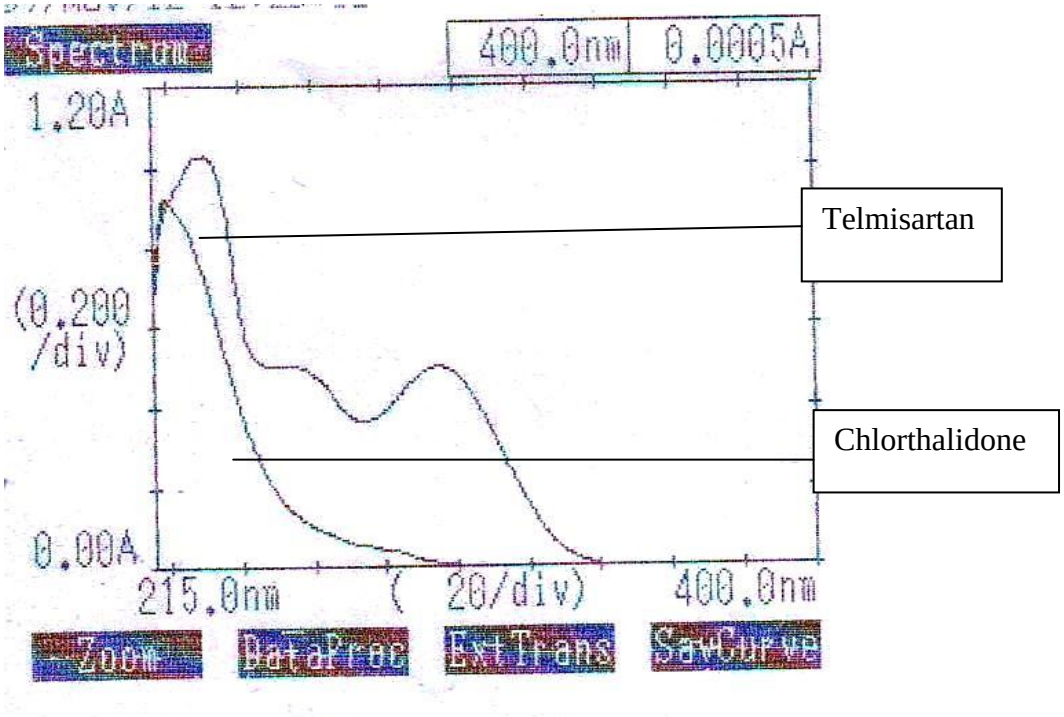


Figure 4

OVERLAIN FIRST ORDER DERIVATIVE SPECTRUM OF TELMISARTAN AND CHLORTHALIDONE

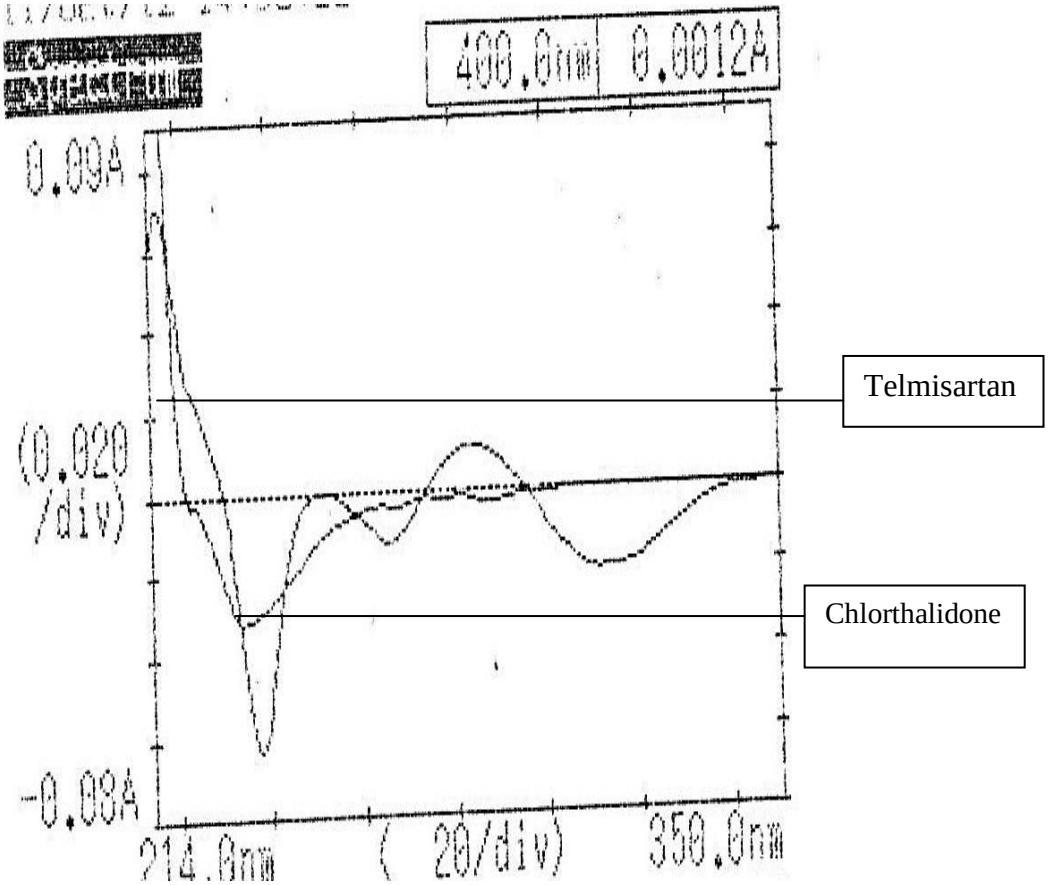


Figure 5

CALIBRATION GRAPH FOR TELMISARTAN AT 311 nm

(FIRST ORDER DERIVATIVE METHOD)

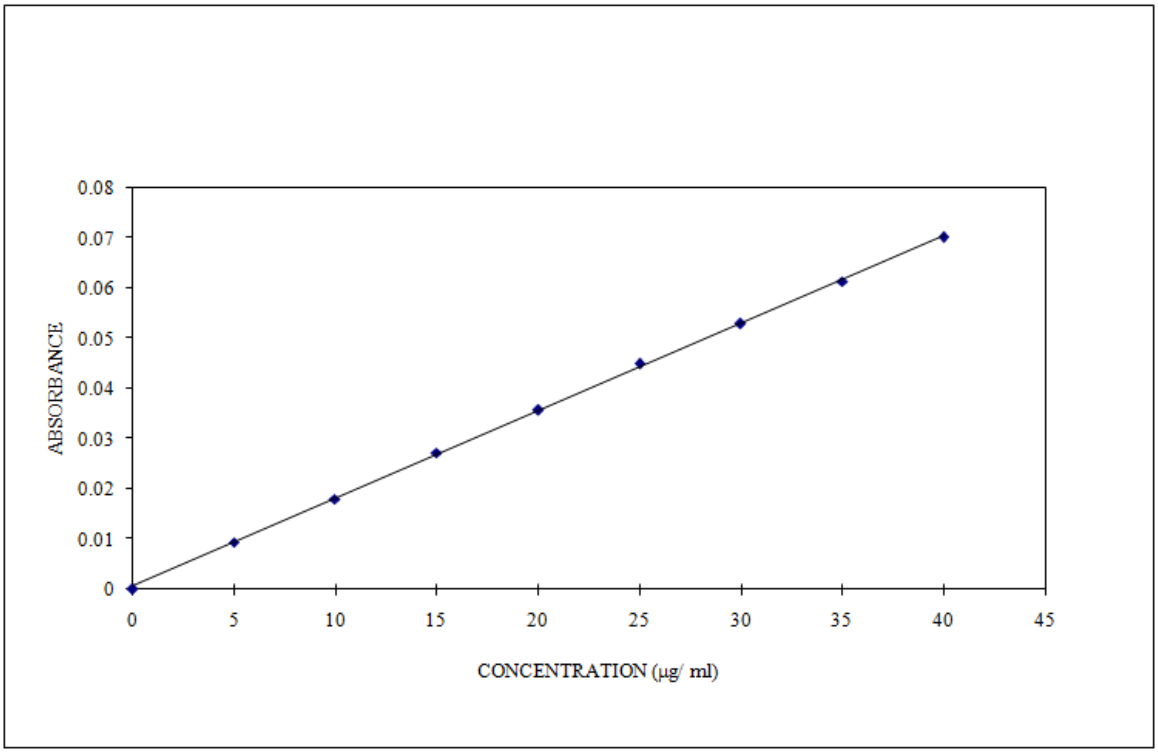


Figure 6

CALIBRATION GRAPH FORCHLORTHALIDONE AT 251 nm

(FIRST ORDER DERIVATIVE METHOD)

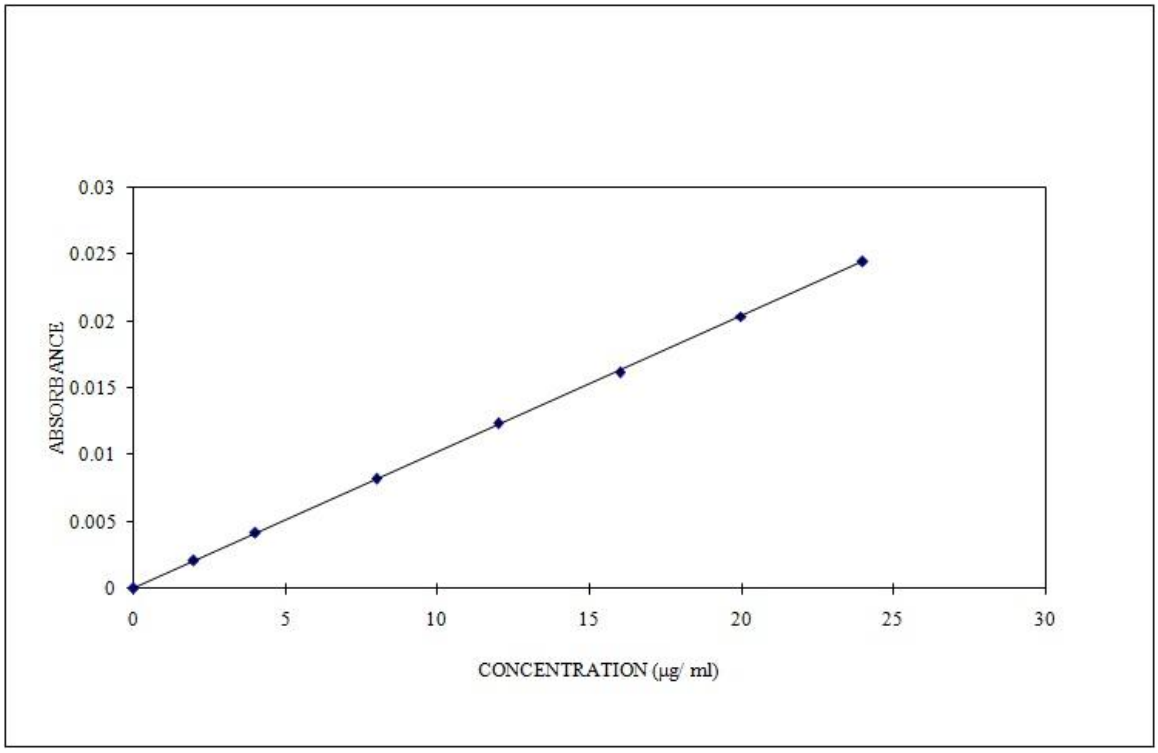


Figure 7

OVERLAIN SPECTRA OF TELMISARTAN AND CHLORTHALIDONE BY HPLC METHOD

(DETECTION WAVELENGTH)

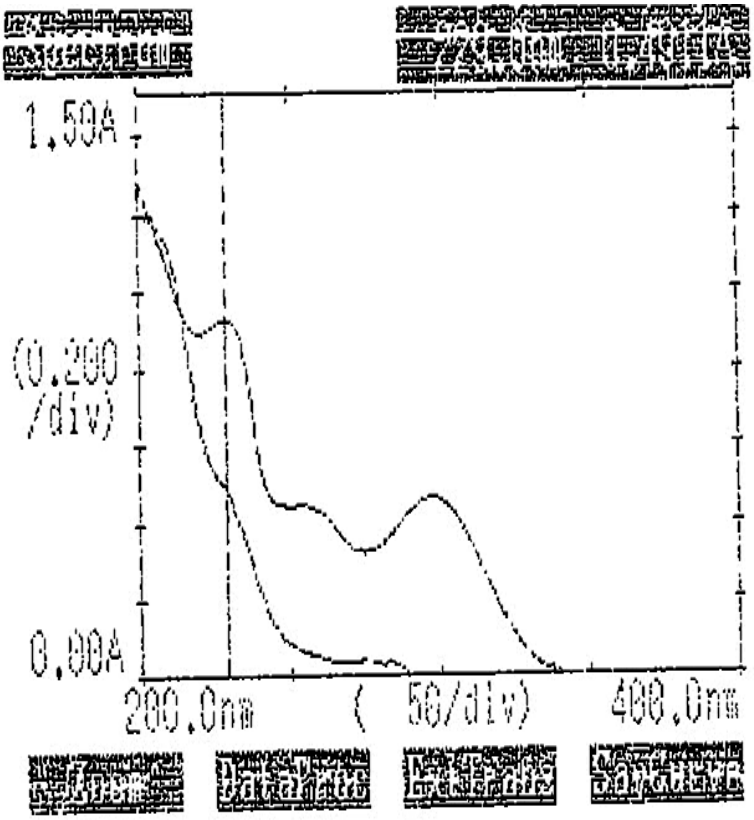


Figure 8

INTIAL SEPARATION CONDITIONS IN

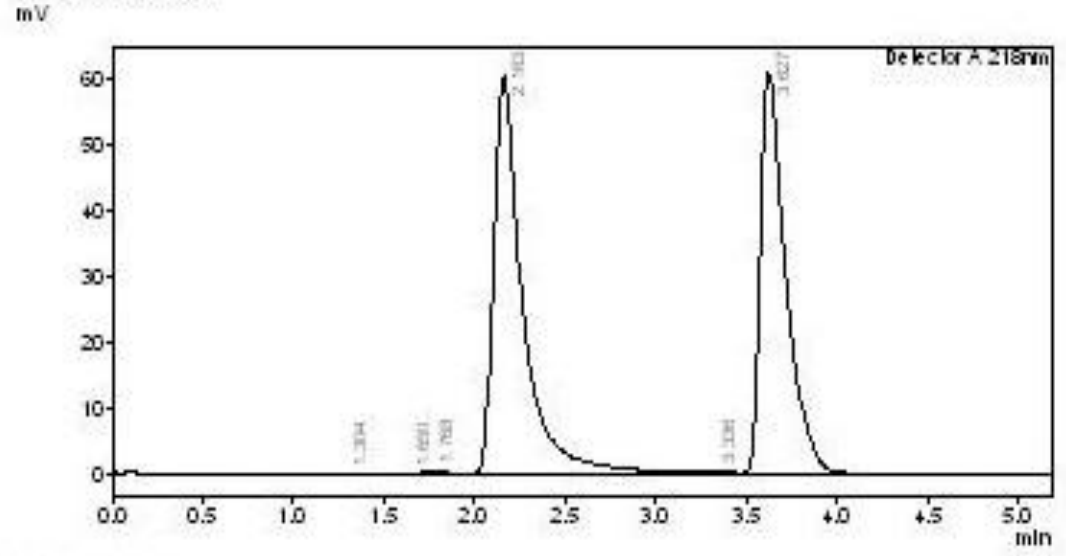
ACETONITRILE: METHANOL (50:50 %v/v)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	:TELCHL		
Sample ID	:TELCHL		
Data Filename	:TELCHL27.lcd		
Method Filename	:TELCHL.lcm		
Batch Filename	:		
Vial #	:-1	Sample Type	:Unknown
Injection Volume	:20 uL		
Date Acquired	:6/2/2013 4:40:48 PM	Acquired by	:user1
Date Processed	:6/3/2013 4:46:00 PM	Processed by	:user1

<Chromatogram>



<Peak Table>

Detector A 218nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	1.304	1811	221	0.000			
2	1.650	1791	293	0.000			
3	1.768	6606	671	0.000		V	
4	2.163	705336	60671	0.000		SV	
5	3.336	1118	162	0.000		T	
6	3.627	604353	61530	0.000		V	
Total		1321015	123548				

Figure 9

INTIAL SEPARATION CONDITIONS IN

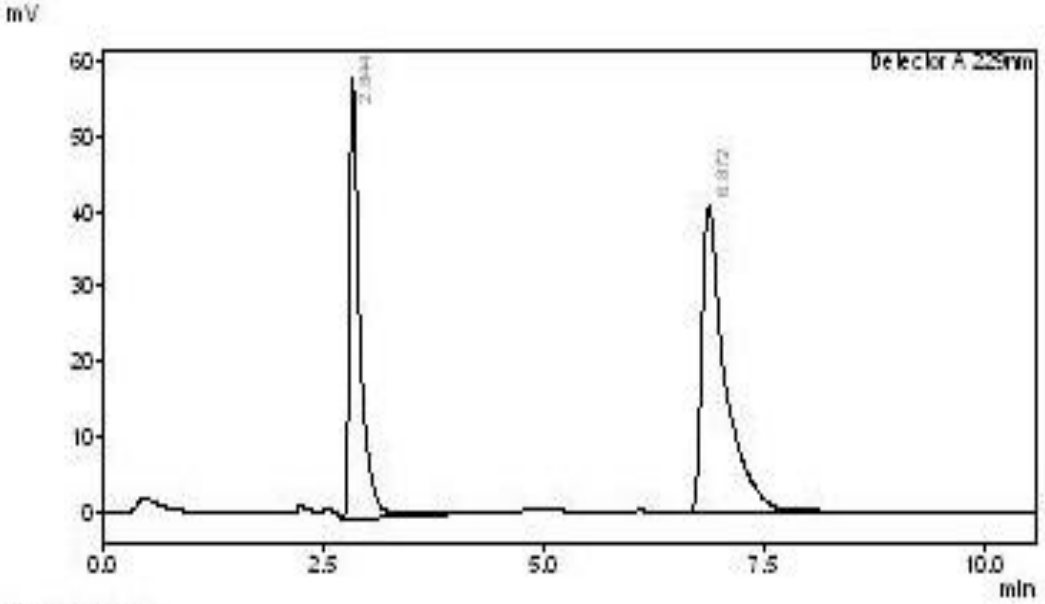
ACETONITRILE: AMMONIUM ACETATE BUFFER (pH 5)(50:50 %v/v)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	:TELCHL		
Sample ID	:TELCHL		
Data Filename	:TELCHL43.lcd		
Method Filename	:TELCHL.lcm		
Batch Filename	:		
Vial #	:-1	Sample Type	: Unknown
Injection Volume	:20 uL		
Date Acquired	:2/21/2013 2:14:54 PM	Acquired by	: user1
Date Processed	:2/21/2013 2:25:29 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.844	494140	58728	40.528		S	
2	6.872	725118	40577	59.472			
Total		1219258	99306				

Figure 10

INTIAL SEPARATION CONDITIONS IN

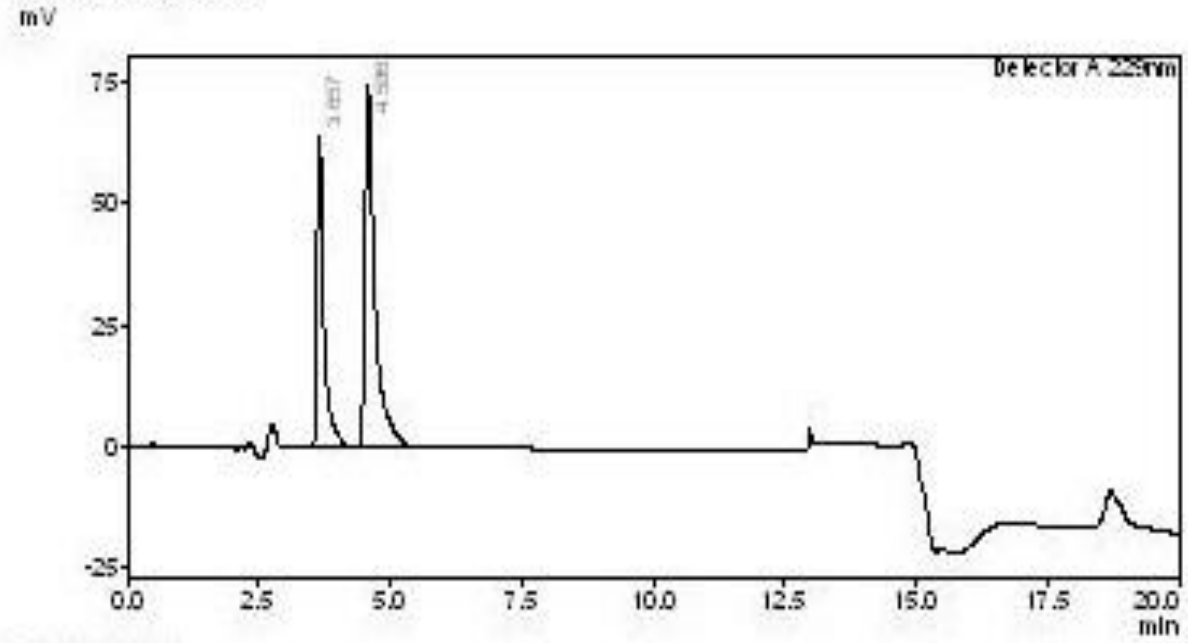
ACETONITRILE: PHOSPHATE BUFFER (pH 3.5)(50:50 %v/v)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL59.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 2/22/2013 4:11:52 PM	Acquired by	: user1
Date Processed	: 2/22/2013 4:31:54 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	3.657	590094	64024	37.164			
2	4.586	997729	74728	62.836		V	
Total		1587823	138752				

Figure 11

INTIAL SEPARATION CONDITIONS IN

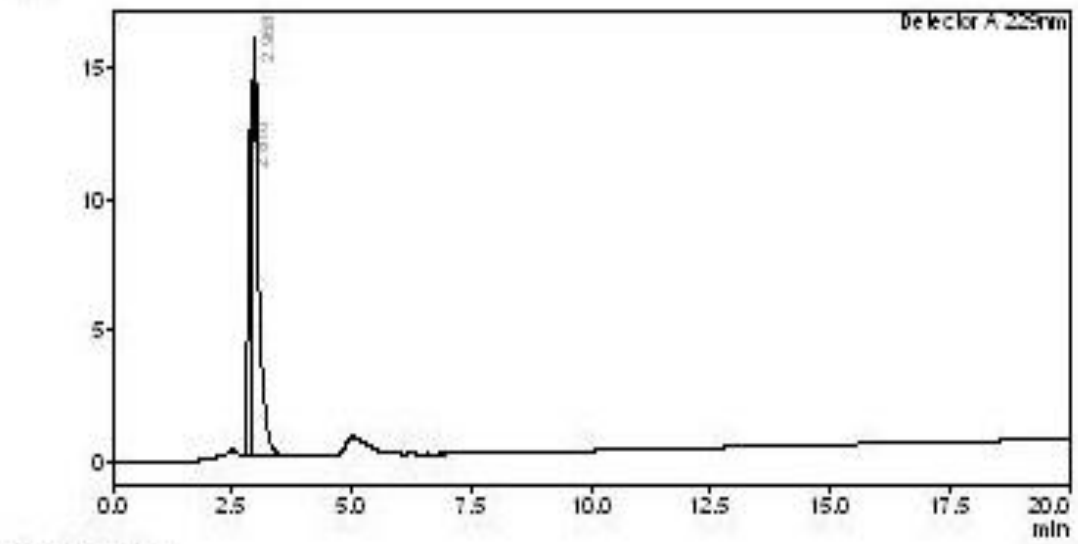
ACETONITRILE: PHOSPHATE BUFFER (pH 3.5)(70:30 %v/v)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL53.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: 1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 2/22/2013 1:27:26 PM	Acquired by	: user1
Date Processed	: 2/22/2013 1:47:28 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.870	50144	10651	24.129			
2	2.968	157675	15995	75.871		V	
Total		207819	26646				

Figure 12

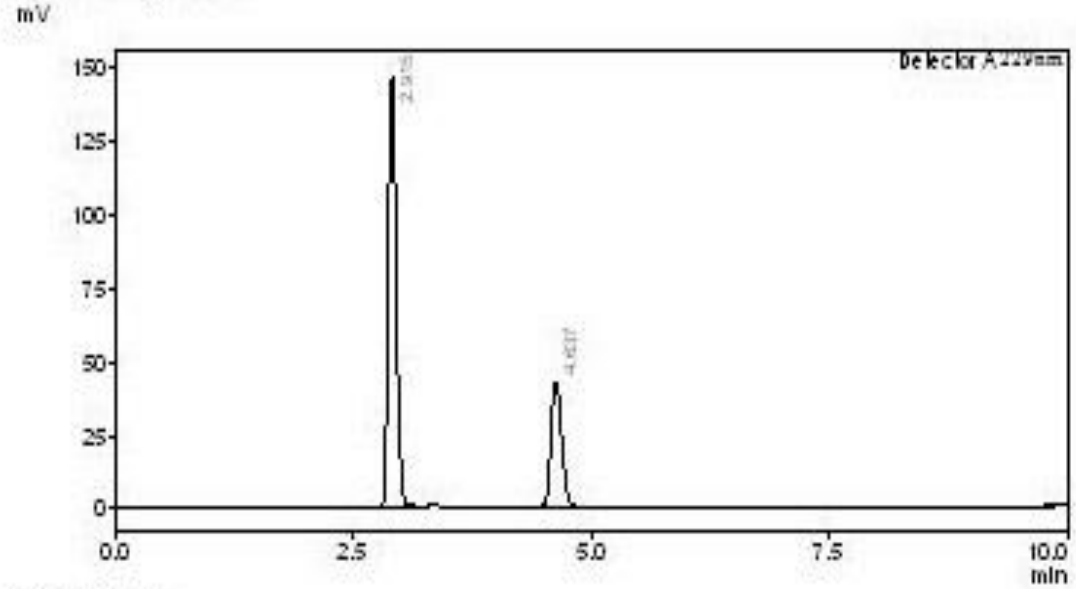
OPTIMIZED CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	:TELCHL		
Sample ID	:TELCHL		
Data Filename	:TELCHL lcd		
Method Filename	:TELCHL.lcm		
Batch Filename	:		
Vial #	:-1	Sample Type	:Unknown
Injection Volume	:20 uL		
Date Acquired	:2/3/2013 2:07:09 PM	Acquired by	:user1
Date Processed	:2/3/2013 3:22:24 PM	Processed by	:user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.915	832350	146926	71.970			
2	4.637	780937	42278	28.030			
Total		1613287	189203				

FIGURE - 13

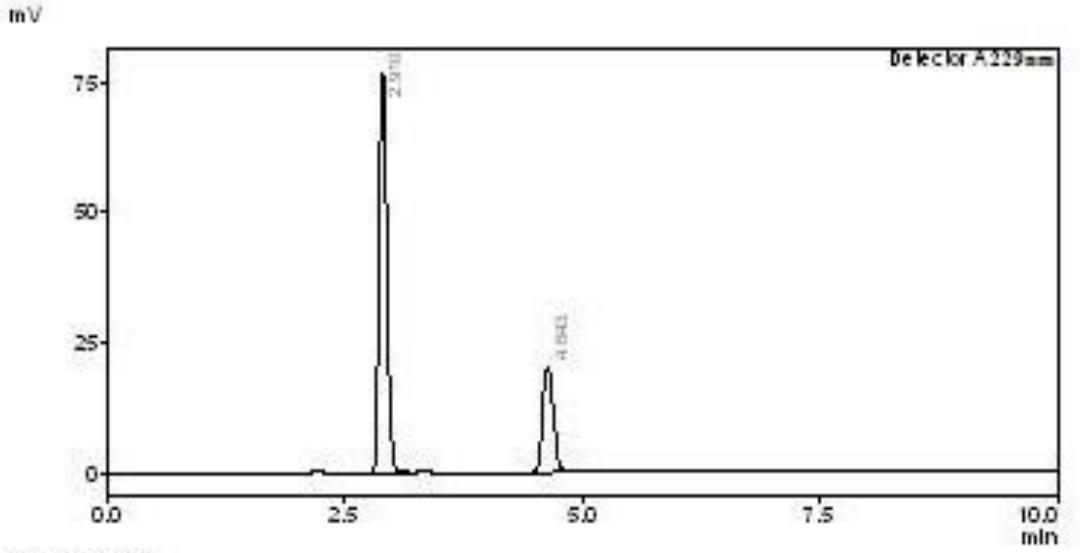
LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE, (5 and 2 µg/ ml)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL 114 2-3.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 2/3/2013 1:56:46 PM	Acquired by	: user1
Date Processed	: 2/3/2013 3:14:32 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.910	433120	76643	73.702			
2	4.643	153245	20266	26.298			
Total		586365	96909				

FIGURE - 14

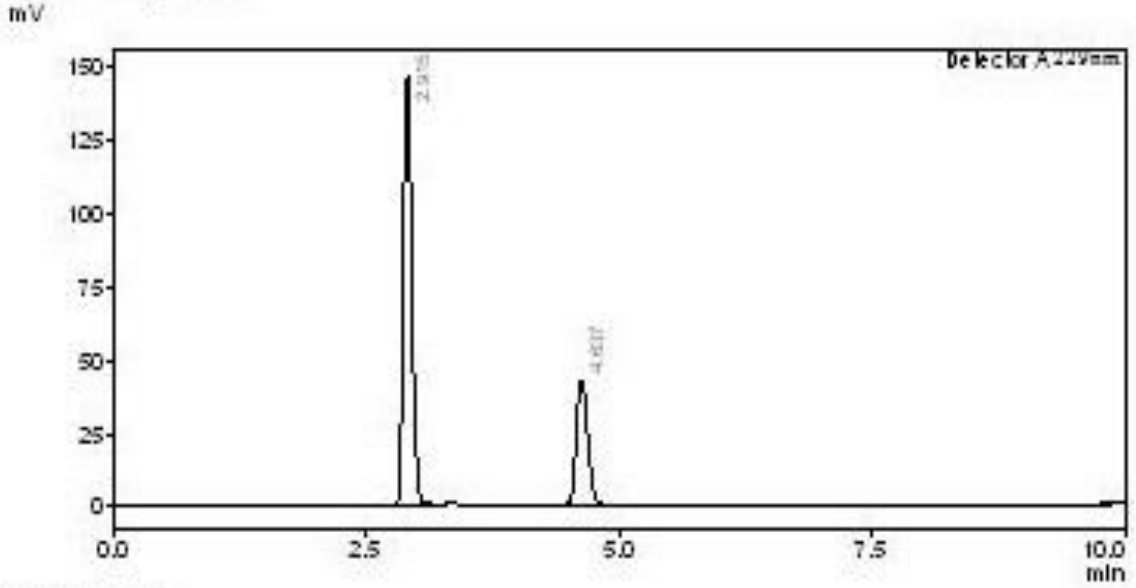
LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (10, 4µg/ ml)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELC HL		
Sample ID	: TELC HL		
Data Filename	: TELC HL 1152-3.lcd		
Method Filename	: TELC HL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 2/3/2013 2:07:09 PM	Acquired by	: user1
Date Processed	: 2/3/2013 3:22:24 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.915	832350	146926	71.970			
2	4.637	312375	42278	28.030			
Total		1144725	189203				

FIGURE - 15

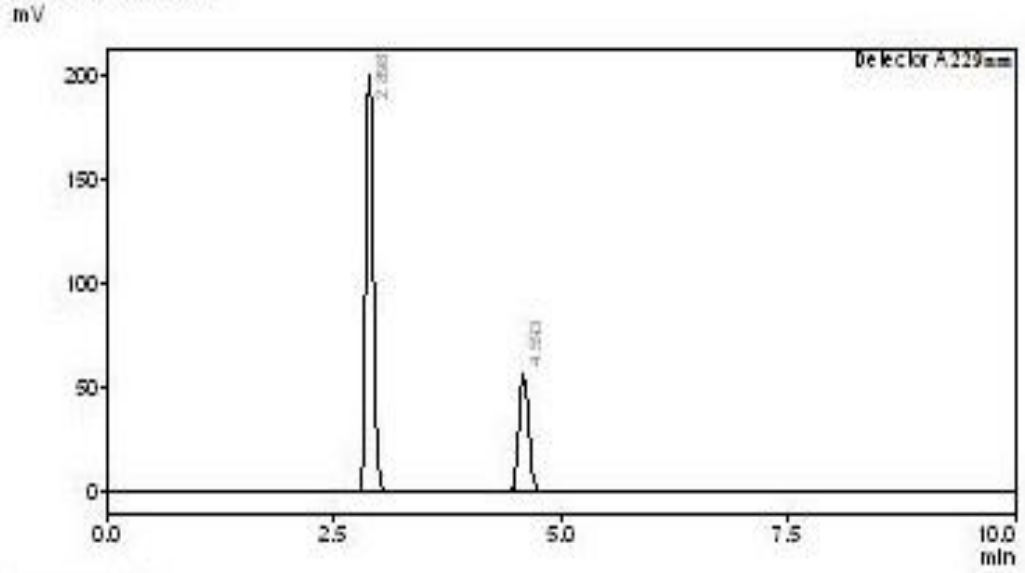
LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (15, 8µg/ ml)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELC HL		
Sample ID	: TELC HL		
Data Filename	: TELC HL 116 2-3.lcd		
Method Filename	: TELC HL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 2/3/2013 2:17:38 PM	Acquired by	: user1
Date Processed	: 2/3/2013 3:25:12 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.896	1244314	200994	72.678			
2	4.593	604836	55914	27.322			
Total		1849150	256908				

FIGURE - 16

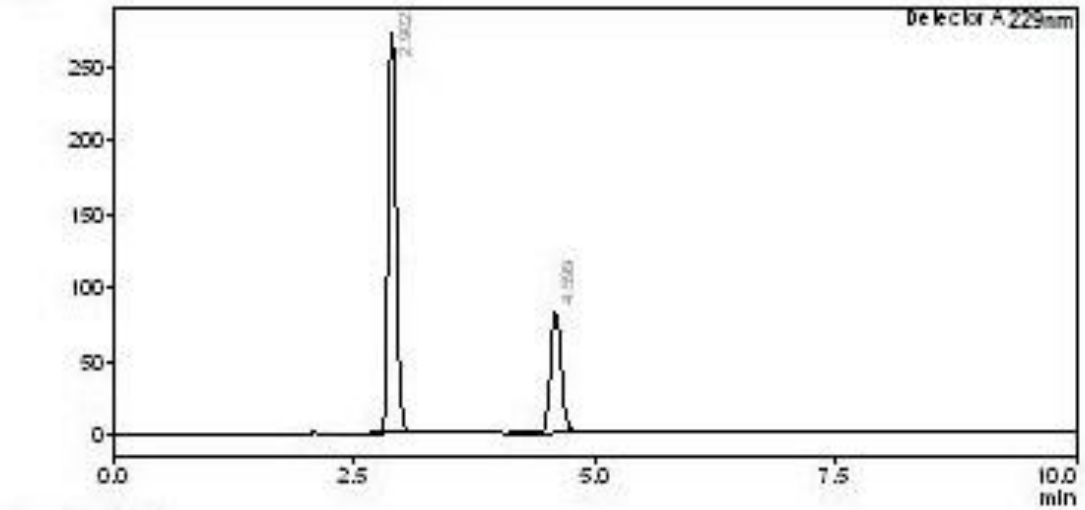
LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (20,12µg/ ml)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	:TELCHL		
Sample ID	:TELCHL		
Data Filename	:TELCHL 117 2-3.lcd		
Method Filename	:TELCHL.lcm		
Batch Filename	:		
Vial #	:-1	Sample Type	: Unknown
Injection Volume	:20 uL		
Date Acquired	:2/3/2013 2:28:28 PM	Acquired by	: user1
Date Processed	:2/3/2013 3:27:18 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.902	1615021	271541	70.737			
2	4.599	905429	80603	29.263			
Total		2520450	352145				

FIGURE - 17

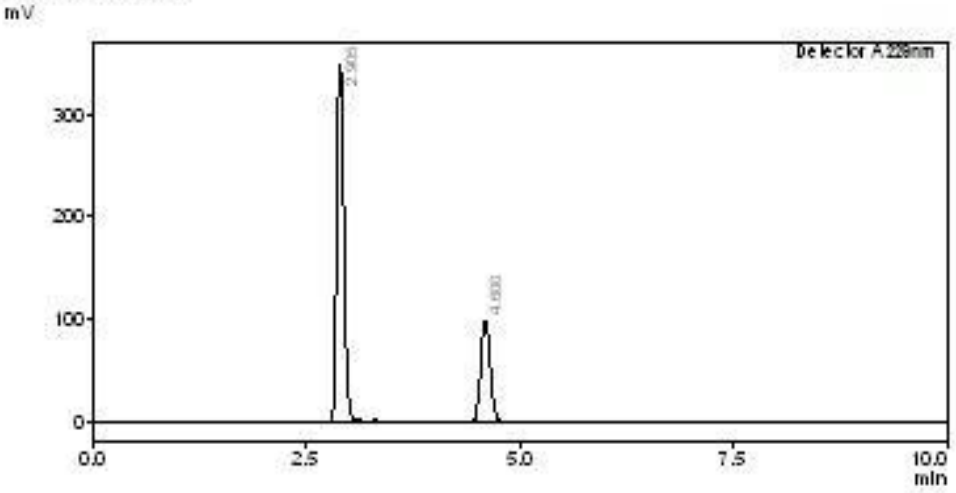
LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE (25, 16µg/ ml)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	:TELCHL		
Sample ID	:TELCHL		
Data Filename	:TELCHL 1182-3.lcd		
Method Filename	:TELCHL.lcm		
Batch Filename	:		
Vial #	:-1	Sample Type	: Unknown
Injection Volume	:20 uL		
Date Acquired	:2/3/2013 2:38:57 PM	Acquired by	: user1
Date Processed	:2/3/2013 3:34:07 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.905	2069152	348556	72.631			
2	4.600	1202116	97294	27.369			
Total		3261268	445850				

FIGURE - 18

CALIBRATION CURVE FOR TELMISARTAN BY RP – HPLC

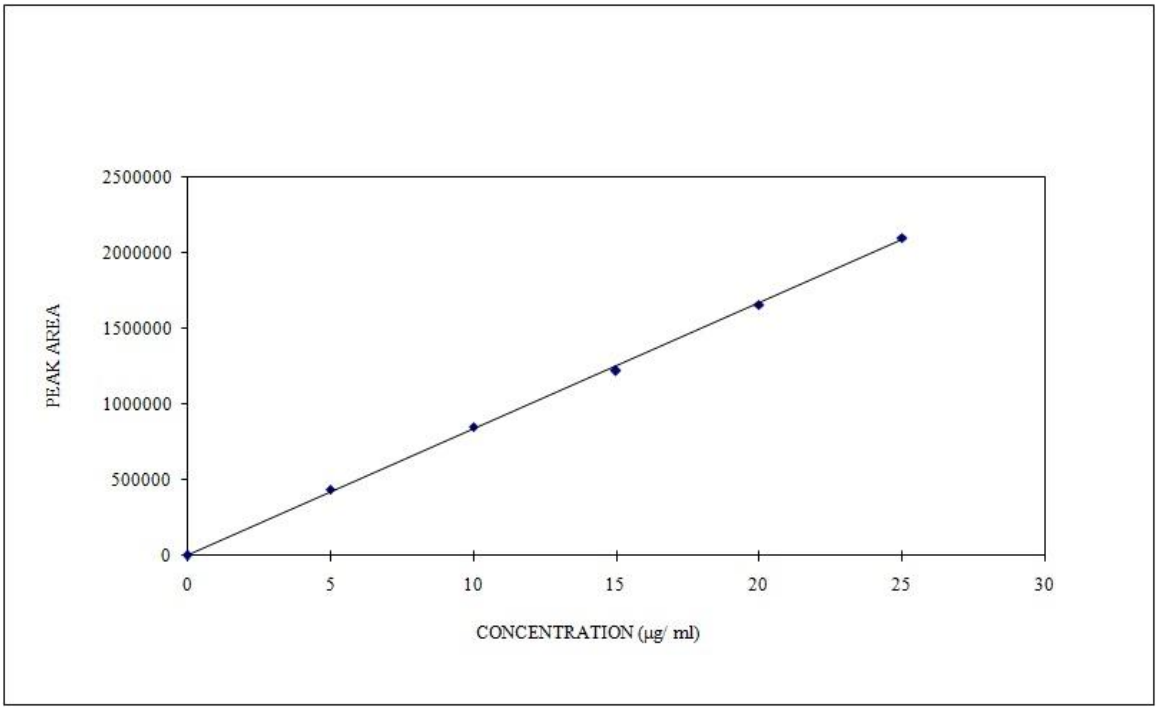


FIGURE - 19

CALIBRATION CURVE FOR CHLORTHALIDONE BY RP – HPLC

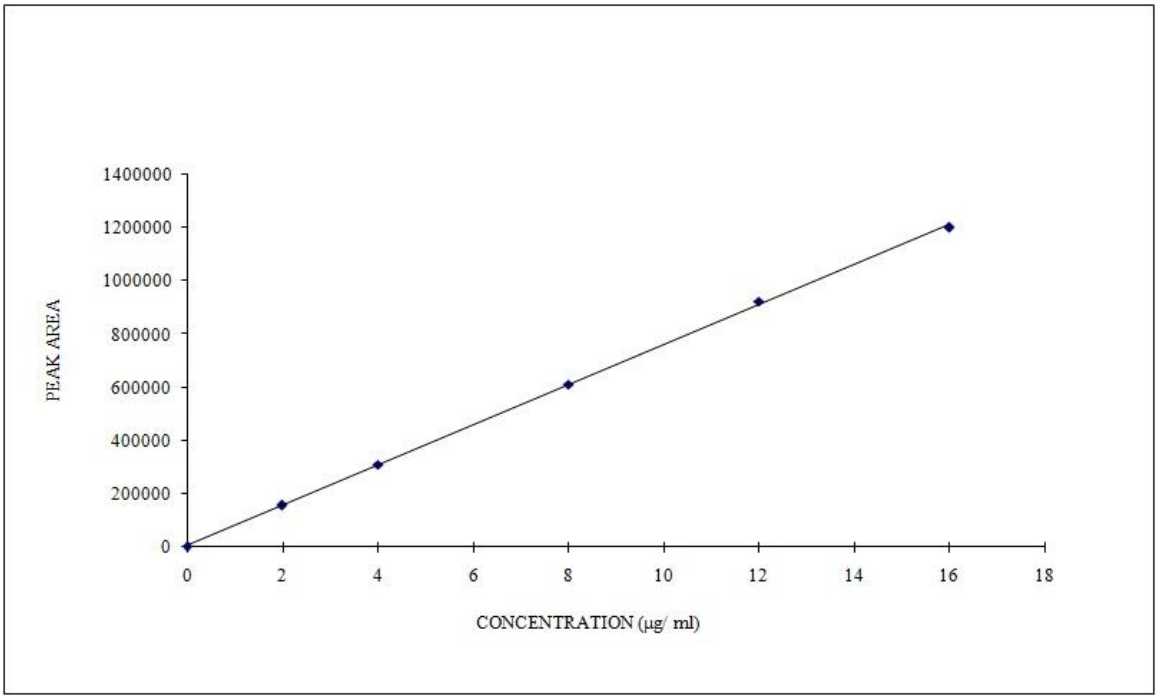


FIGURE-20

CHROMATOGRAM FOR ANALYSIS OF FORMULATION – ERITEL CH 40

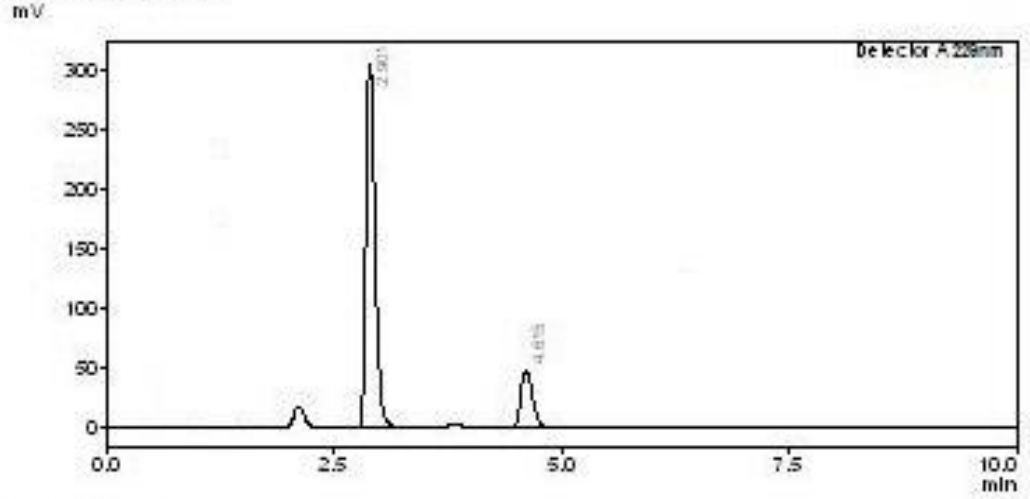
REPEATABILITY – 1

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELC HL		
Sample ID	: TELC HL		
Data Filename	: TELC HL 119.lcd		
Method Filename	: TELC HL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 4/3/2013 1:48:15 PM	Acquired by	: user1
Date Processed	: 4/3/2013 3:38:50 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	0.099	1684	285	0.066			
2	2.901	684016	304585	83.086		S	
3	4.615	197286	46763	16.848		S	
Total		882986	351633				

FIGURE-21

CHROMATOGRAM FOR ANALYSIS OF FORMULATION – ERITEL CH 40

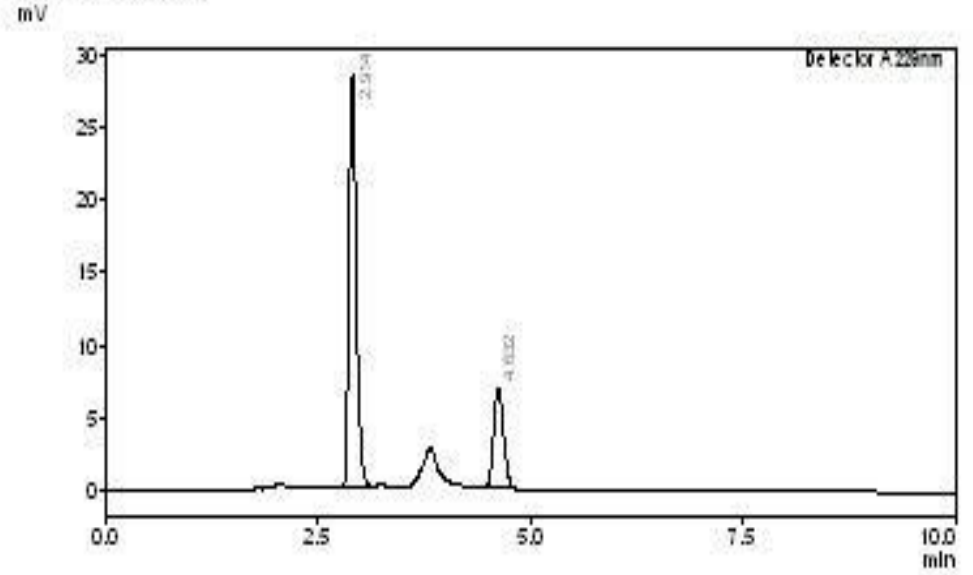
REPEATABILITY –2

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL 120.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: 1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 4/3/2013 1:58:31 PM	Acquired by	: user1
Date Processed	: 4/3/2013 3:45:11 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.914	680116	28504	75.436			
2	4.632	196051	6829	24.564		S	
Total		876167	35333				

FIGURE-22

CHROMATOGRAM FOR ANALYIS OF FORMULATION – ERITEL CH 40

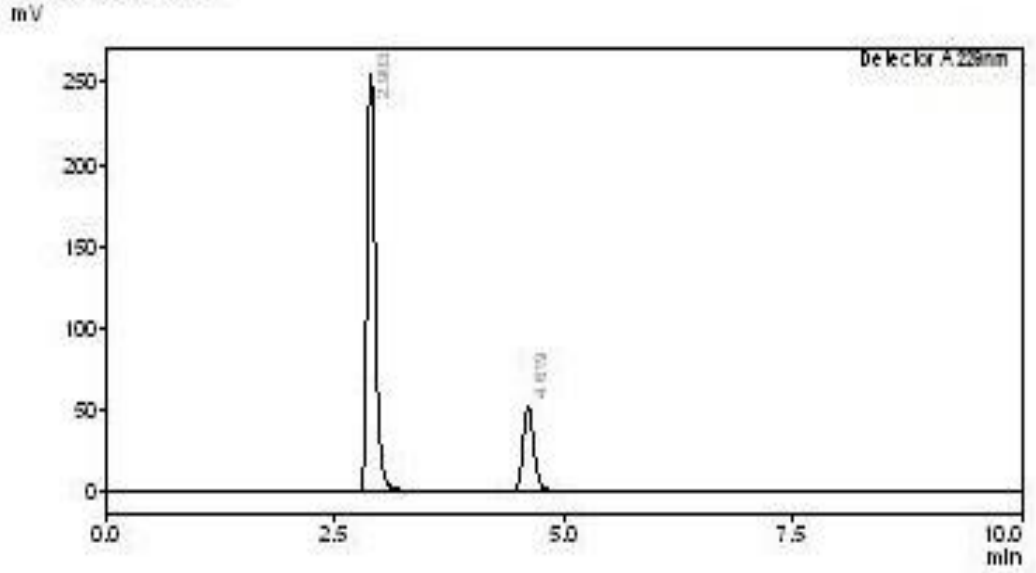
REPEATABILITY – 3

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELC HL		
Sample ID	: TELC HL		
Data Filename	: TELC HL 121.lcd		
Method Filename	: TELC HL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 µL		
Date Acquired	: 4/3/2013 2:21:19 PM	Acquired by	: user1
Date Processed	: 4/3/2013 3:47:50 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.903	680998	255708	77.731		S	
2	4.619	198950	51517	22.269			
Total		879948	307225				

FIGURE-23

CHROMATOGRAM FOR ANALYSIS OF FORMULATION – ERITEL CH 40

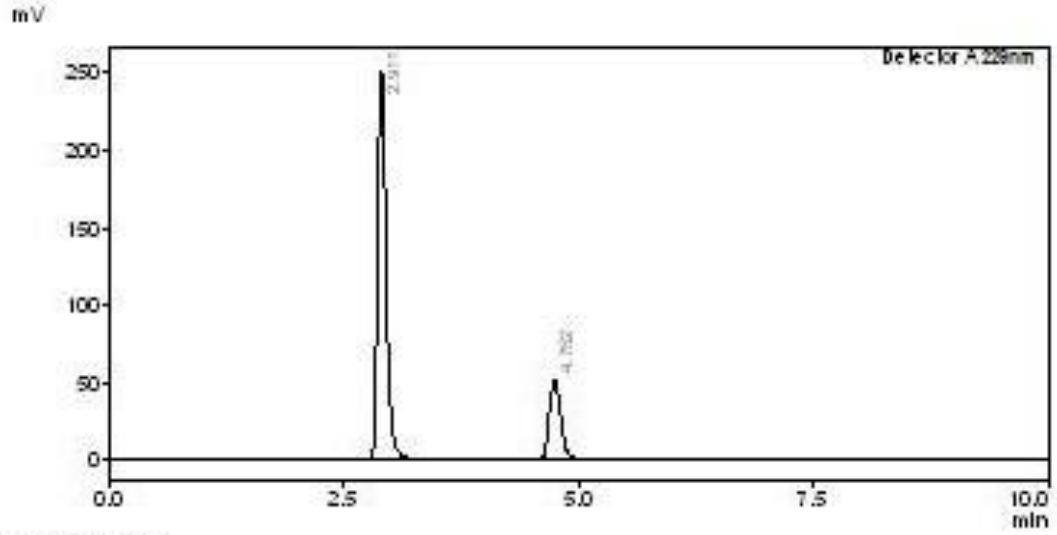
REPEATABILITY – 4

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELC HL		
Sample ID	: TELC HL		
Data Filename	: TELC HL 122.lcd		
Method Filename	: TELC HL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 4/3/2013 2:31:39 PM	Acquired by	: user1
Date Processed	: 4/3/2013 3:49:47 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.911	680152	251414	78.221		S	
2	4.752	196691	51496	21.779			
Total		876843	302910				

FIGURE-24

CHROMATOGRAM FOR ANALYSIS OF FORMULATION – ERITEL CH 40

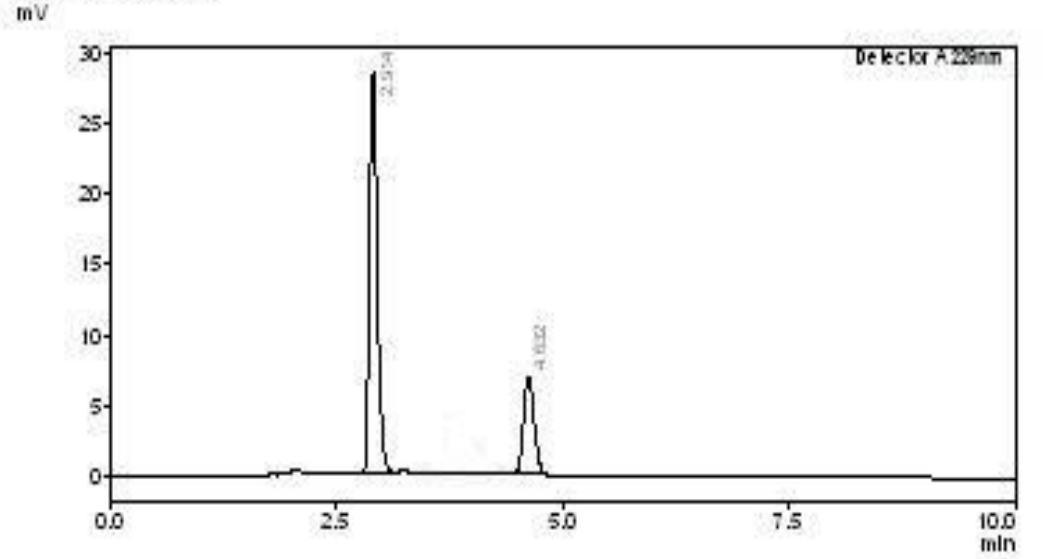
REPEATABILITY – 5

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL 123.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 4/3/2013 1:58:31 PM	Acquired by	: user1
Date Processed	: 4/3/2013 3:45:11 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.914	680577	28504	75.436			
2	4.632	198021	6231	24.564		S	
Total		878598	34735				

FIGURE-25

CHROMATOGRAM FOR ANALYSIS OF FORMULATION – ERITEL CH 40

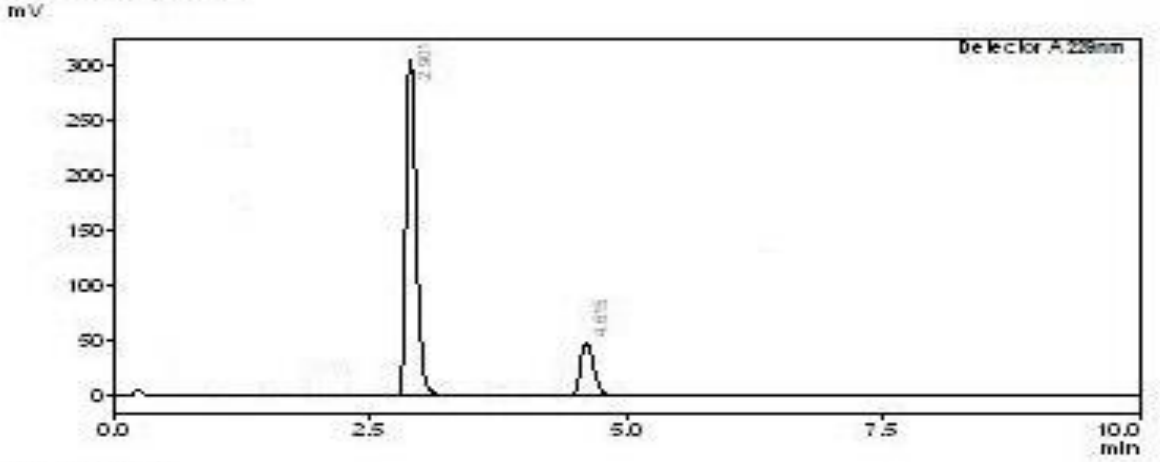
REPEATABILITY – 6

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELC HL		
Sample ID	: TELC HL		
Data Filename	: TELC HL124.lcd		
Method Filename	: TELC HL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 µL		
Date Acquired	: 4/3/2013 1:48:15 PM	Acquired by	: user1
Date Processed	: 4/3/2013 3:38:50 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 229nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	0.099	1684	285	0.066			
2	2.901	692051	314585	83.086		S	
3	4.615	196721	43251	16.848		S	
Total		890772	358121				

FIGURE-26

CHROMATOGRAM FOR FIRST RECOVERY OF FORMULATION - 1

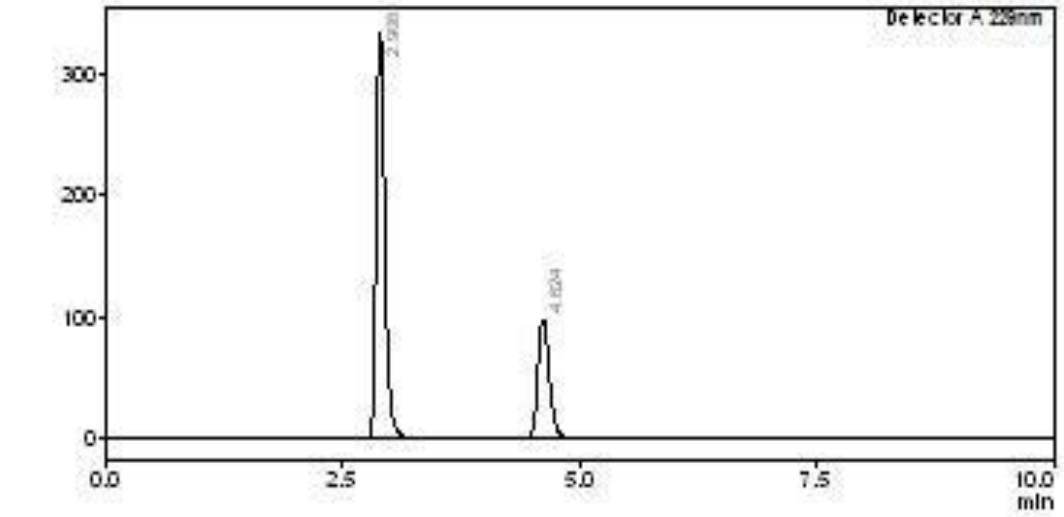
(ERITEL CH- 40)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	:TELCHL		
Sample ID	:TELCHL		
Data Filename	:TELCHL127 1.lcd		
Method Filename	:TELCHL.lcm		
Batch Filename	:		
Vial #	:-1	Sample Type	: Unknown
Injection Volume	:20 uL		
Date Acquired	:5/3/2013 2:42:00 PM	Acquired by	: user1
Date Processed	:5/3/2013 3:52:27 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.906	1212412	334427	71.380			
2	4.624	352421	97197	28.620			
Total		1564833	431624				

FIGURE-27

CHROMATOGRAM FOR FIRST RECOVERY OF FORMULATION - 2

(ERITEL CH- 40)

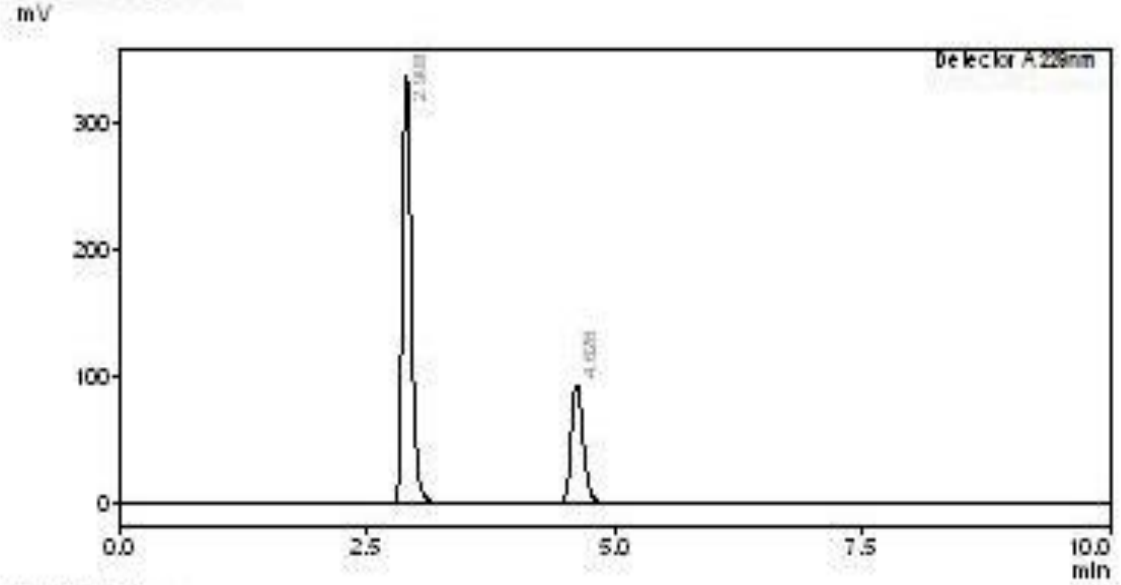
SHIMADZU LabSolutions

Analysis Report

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL 1292.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 5/3/2013 2:52:29 PM	Acquired by	: user1
Date Processed	: 5/3/2013 3:54:47 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.908	1345212	336367	72.218			
2	4.625	385210	93662	27.782			
Total		1730422	430030				

FIGURE-28

CHROMATOGRAM FOR FIRST RECOVERY OF FORMULATION - 3

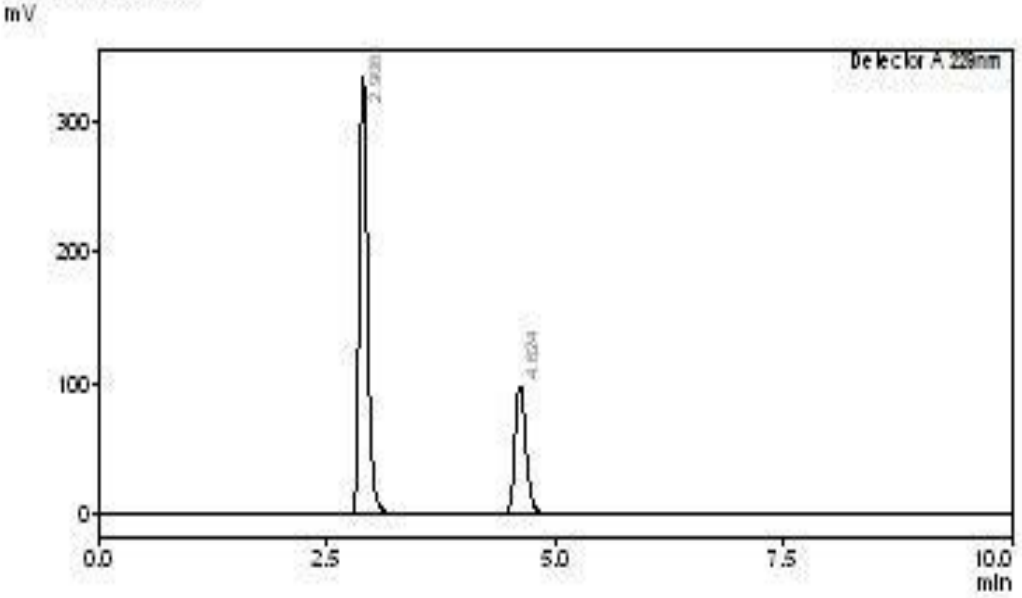
(ERITEL CH- 40)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: TELCHL		
Sample ID	: TELCHL		
Data Filename	: TELCHL 1313.lcd		
Method Filename	: TELCHL.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 5/3/2013 2:42:00 PM	Acquired by	: user1
Date Processed	: 5/3/2013 3:52:27 PM	Processed by	: user1

<Chromatogram>



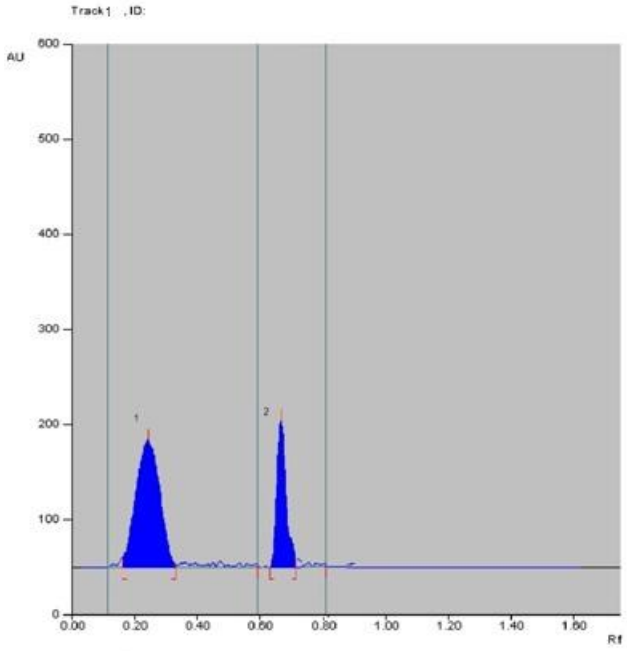
<Peak Table>

Detector A 228nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	2.906	1497076	312120	71.380			
2	4.624	427520	95182	28.620			
Total		1924596	407302				

Figure -29

OPTIMIZED CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC

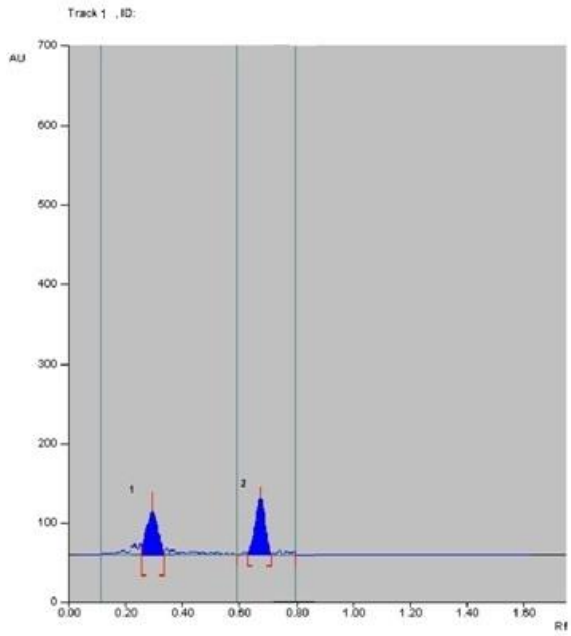


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.26 Rf	6.8 AU	0.29 Rf	29.2 AU	100.00 %	0.34 Rf	1.7 AU	415.3 AU	100.00 %	Telmisartan *
2	0.62 Rf	0.2 AU	0.65 Rf	61.4 AU	100.00 %	0.69 Rf	0.7 AU	792.1 AU	100.00 %	Chlorthalidone *

FIGURE-30

LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC

(100 + 50 ng/ µl)

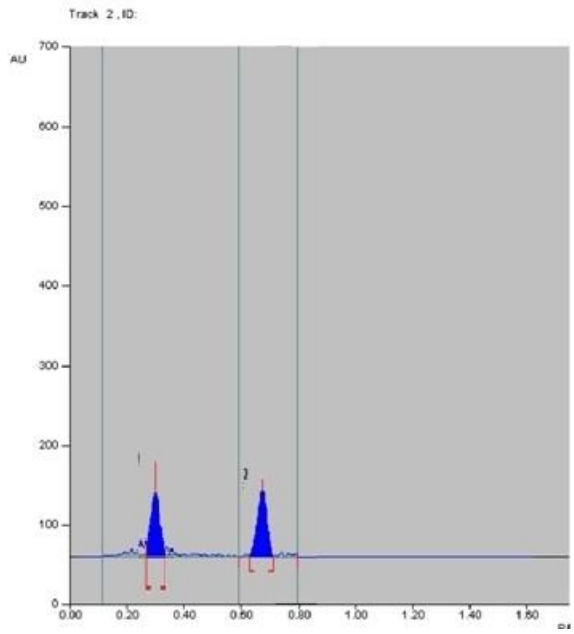


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.22 Rf	6.0 AU	0.28 Rf	98.4 AU	100.00 %	0.33 Rf	8.0 AU	213.2 AU	100.00 %	Telmisartan *
2	0.62 Rf	0.1 AU	0.65 Rf	35.1 AU	100.00 %	0.68 Rf	0.0 AU	507.1 AU	100.00 %	Chlorthalidone *

FIGURE-31

LINEARITY CHROMATOGRAM FOR TELMISARTAN AND CHLORTHALIDONE BY HPTLC

(200 + 100 ng/ µl)

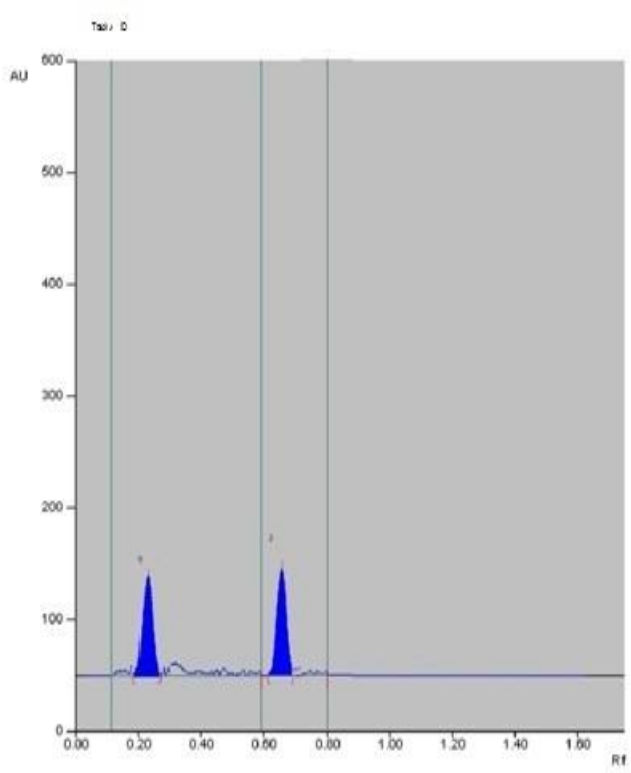


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.26 Rf	6.8 AU	0.29 Rf	29.2 AU	100.00 %	0.34 Rf	1.7 AU	415.3 AU	100.00 %	Telmisartan *
2	0.62 Rf	0.2 AU	0.65 Rf	61.4 AU	100.00 %	0.69 Rf	0.7 AU	792.1 AU	100.00 %	Chlorthalidone *

FIGURE-32

LINEARITY CHROMATOGRAM FORTELMISARTAN AND CHLORTHALIDONE BY HPTLC

(300 + 150 ng/ µl)

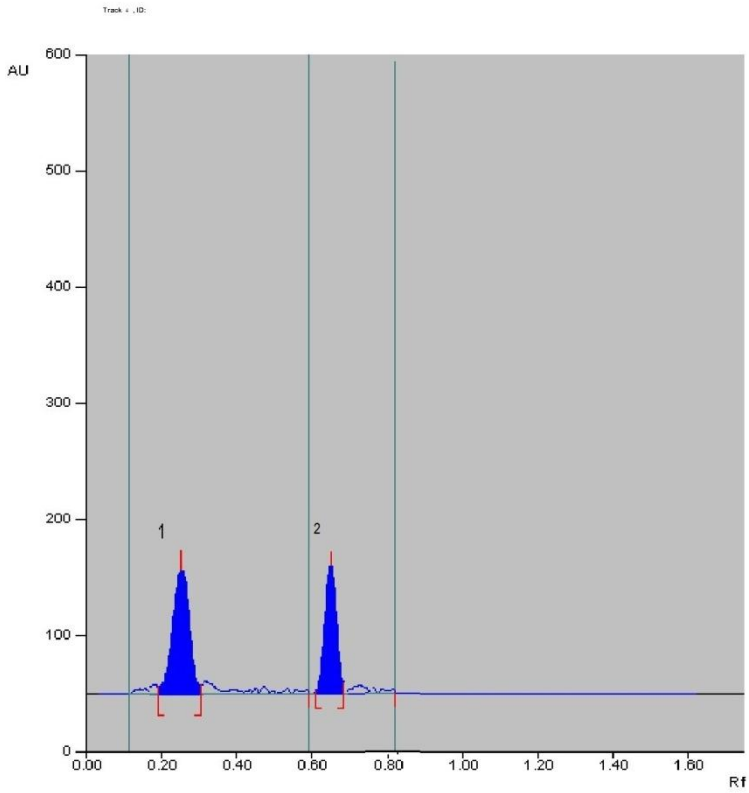


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.22 Rf	3.9 AU	0.27 Rf	81.8 AU	100.00 %	0.31 Rf	0.3 AU	584.9 AU	100.00 %	Telmisartan*
2	0.63 Rf	1.4 AU	0.65 Rf	77.9 AU	100.00 %	0.67 Rf	0.3 AU	1196.4 AU	100.00 %	Chlorthalidone *

FIGURE-33

LINEARITY CHROMATOGRAM FORTELMISARTAN AND CHLORTHALIDONE BY HPTLC

(400 + 200 ng/ µl)

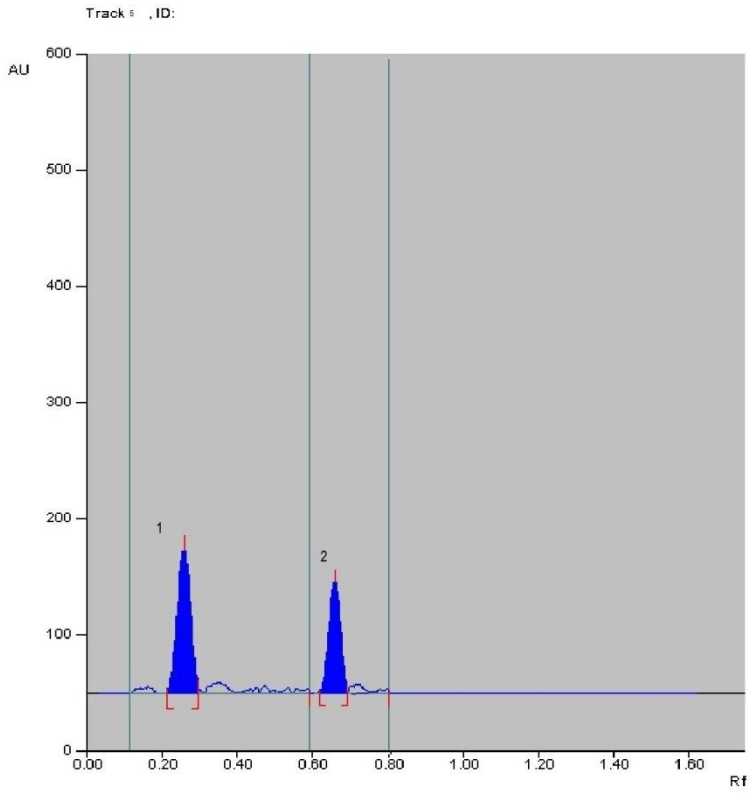


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.24 Rf	6.9 AU	0.28 Rf	19.8 AU	100.00 %	0.34 Rf	0.6 AU	797.8 AU	100.00 %	Telmisartan*
2	0.62 Rf	2.5 AU	0.64 Rf	110.9 AU	100.00 %	0.69 Rf	10.0 AU	1471.6 AU	100.00 %	Chlorthalidone*

FIGURE-34

LINEARITY CHROMATOGRAM FORTELMISARTAN AND CHLORTHALIDONE BY HPTLC

(500 + 250 ng/ µl)

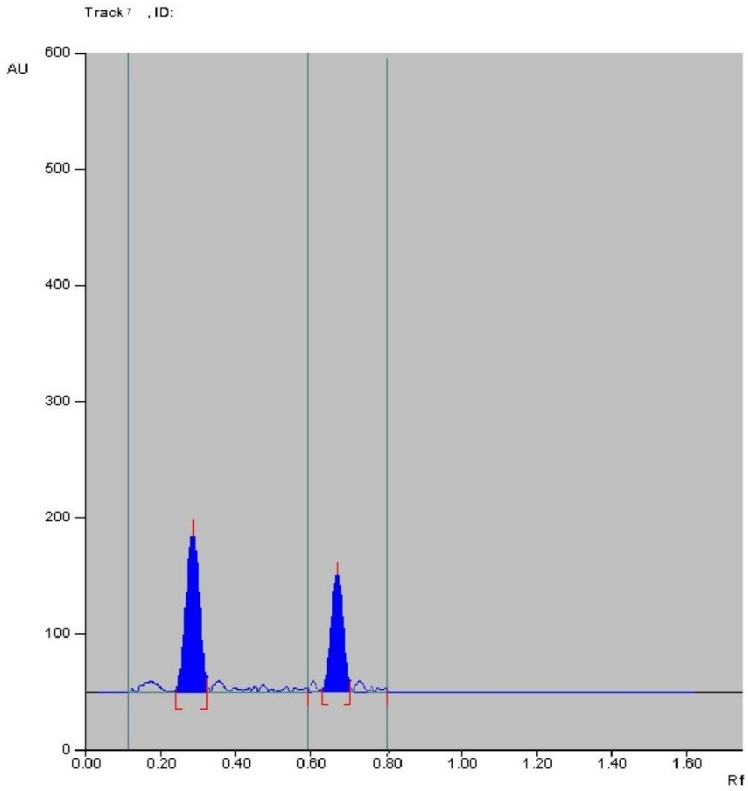


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.21 Rf	5.4 AU	0.29 Rf	52.8 AU	100.00 %	0.31 Rf	0.5 AU	1018.9 AU	100.00 %	Telmisartan *
2	0.64 Rf	0.2 AU	0.66 Rf	140.7 AU	100.00 %	0.69 Rf	18.4 AU	1821.5 AU	100.00 %	Chlorthalidone*

FIGURE-35

LINEARITY CHROMATOGRAM FORTELMISARTAN AND CHLORTHALIDONE BY HPTLC

(600 + 300 ng/ µl)

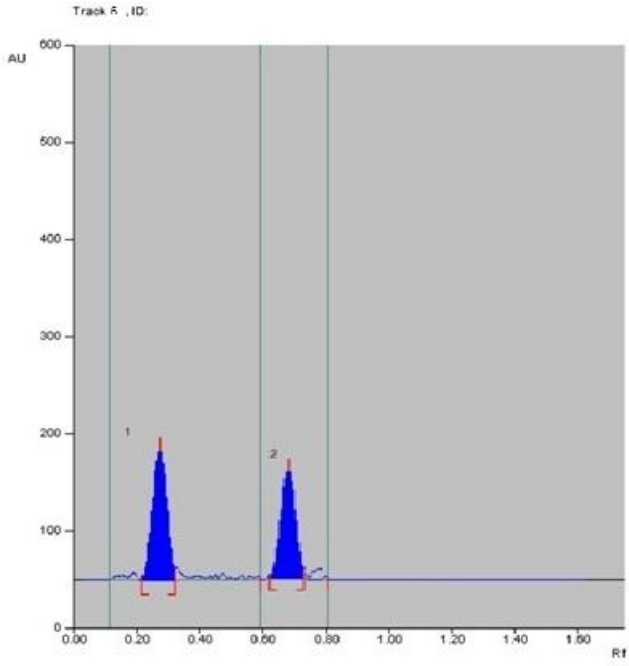


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.19 Rf	3.9 AU	0.28 Rf	81.8 AU	100.00 %	0.39 Rf	0.3 AU	1272.7 AU	100.00 %	Telmisartan *
2	0.63 Rf	1.2 AU	0.65 Rf	174.8 AU	100.00 %	0.67 Rf	12.6 AU	2197.1 AU	100.00 %	Chlorthalidone *

FIGURE-36

LINEARITY CHROMATOGRAM FORTELMISARTAN AND CHLORTHALIDONE BY HPTLC

(700 + 350 ng/ µl)

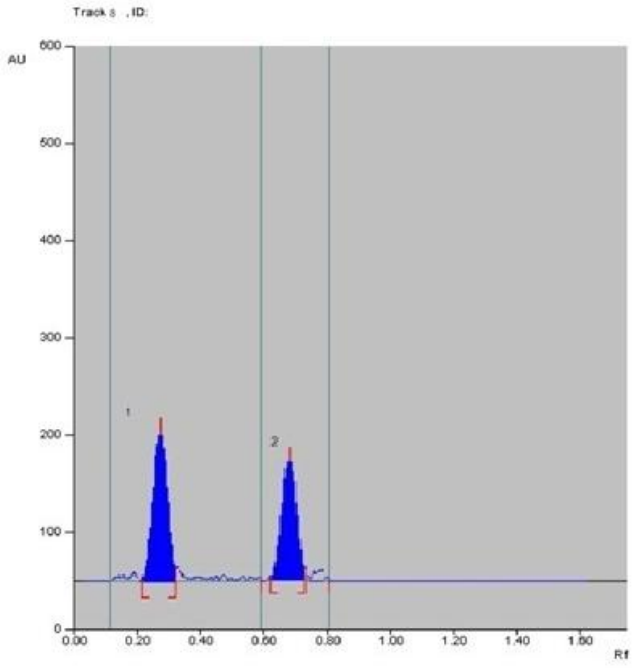


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.20 Rf	7.4 AU	0.27 Rf	111.7 AU	100.00 %	0.32 Rf	3.1 AU	1479.6 AU	100.00 %	Telmisartan *
2	0.63 Rf	0.1 AU	0.65 Rf	154.9 AU	100.00 %	0.69 Rf	7.0 AU	2565.4 AU	100.00 %	Chlorthalidone *

FIGURE-37

LINEARITY CHROMATOGRAM FORTELMISARTAN AND CHLORTHALIDONE BY HPTLC

(800 + 400 ng/ µl)



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.20 Rf	8.1 AU	0.27 Rf	122.2 AU	100.00 %	0.36 Rf	2.2 AU	1690.8 AU	100.00 %	Telmisartan *
2	0.63 Rf	0.3 AU	0.65 Rf	108.9 AU	100.00 %	0.67 Rf	0.0 AU	2890.6 AU	100.00 %	Chlorthalidone *

FIGURE-38

CALIBRATION GRAPH FOR TELMISARTAN BY HPTLC

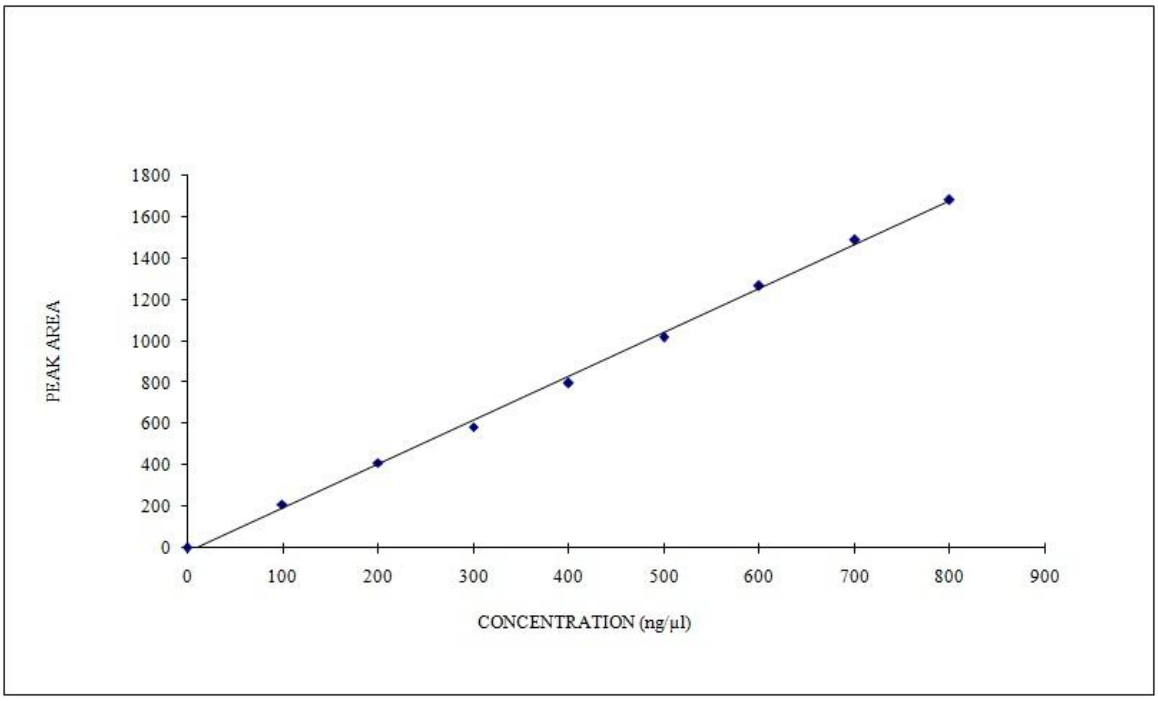


FIGURE-39

CALIBRATION GRAPH FOR CHLORTHALIDONE BY HPTLC

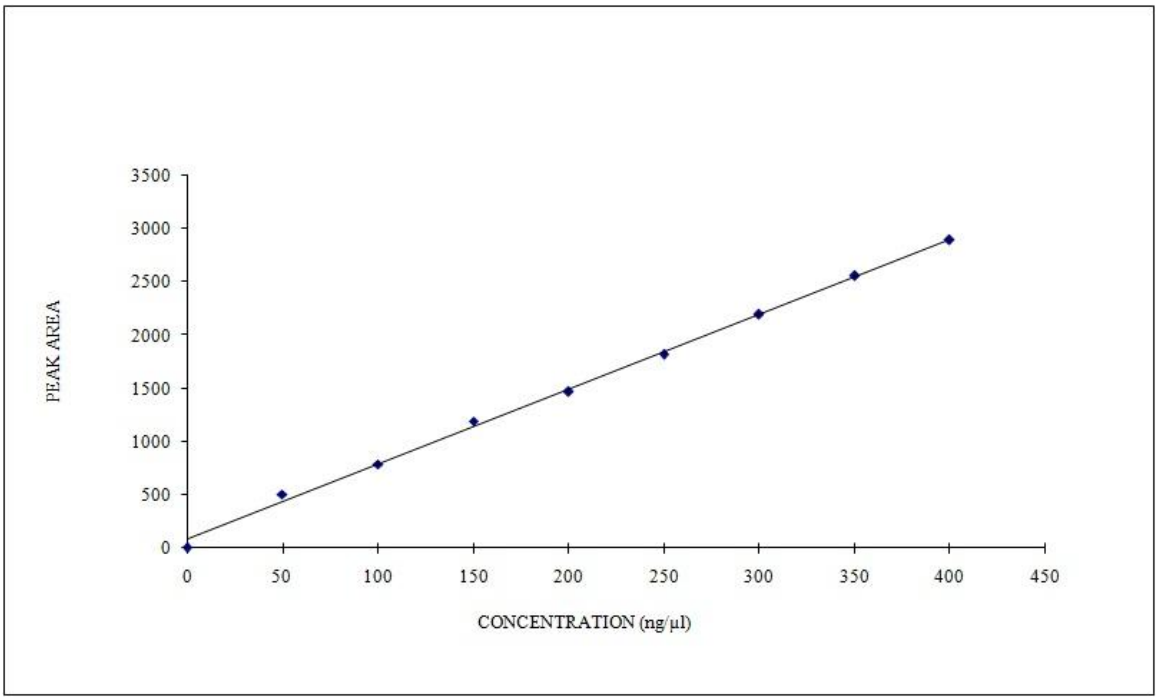
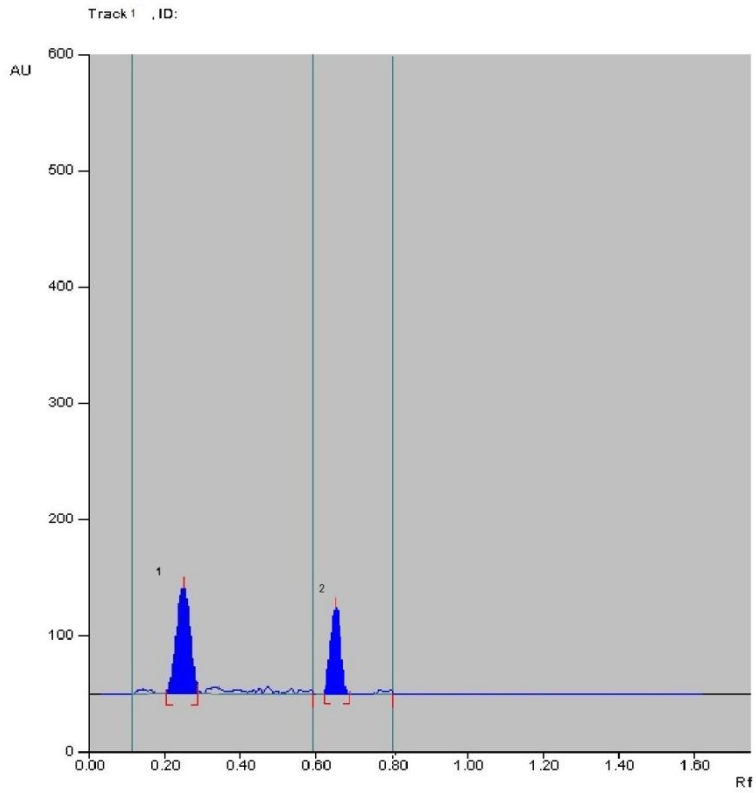


FIGURE-40

CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC

REPEATABILITY 1

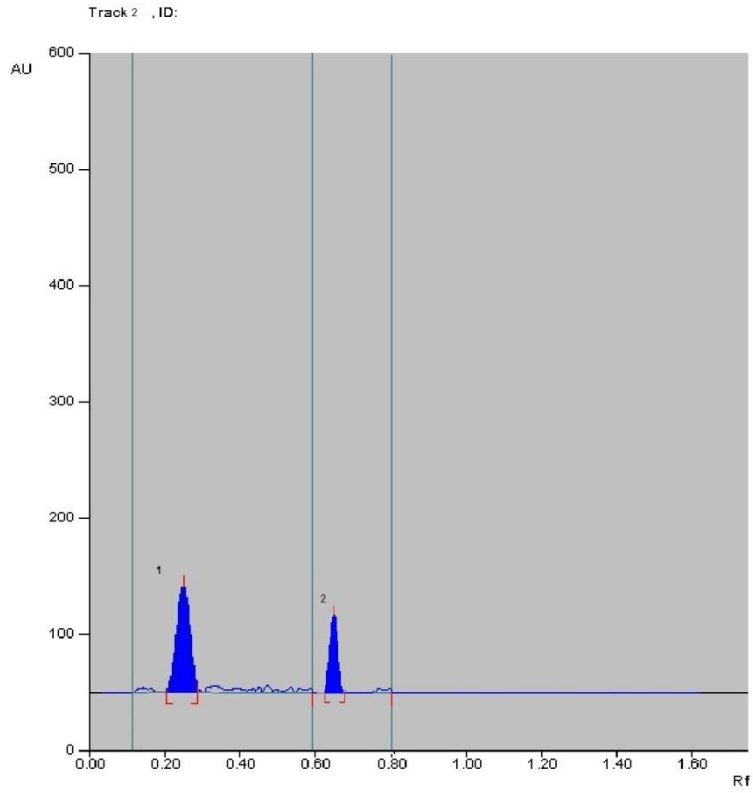


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.22 Rf	12.5 AU	0.28 Rf	30.7 AU	91.43 %	0.38 Rf	9.2 AU	676.13 AU	97.90 %	Telmisartan*
2	0.63 Rf	0.6 AU	0.65 Rf	43.4 AU	98.57 %	0.69 Rf	1.1 AU	799.7 AU	98.10 %	Chlorthalidone *

FIGURE-41

CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC

REPEATABILITY -2

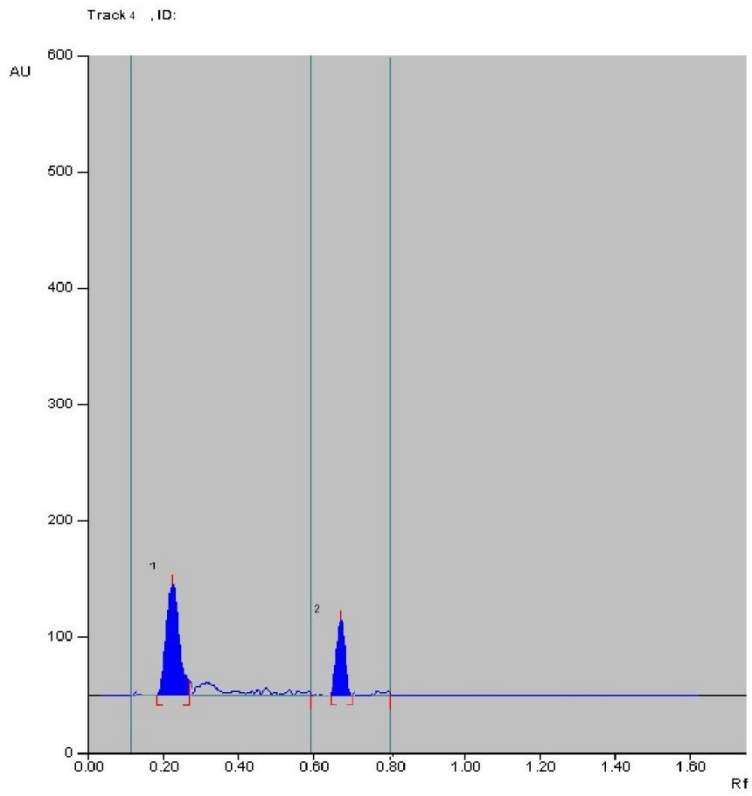


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.22 Rf	3.9 AU	0.27 Rf	81.8 AU	100.00 %	0.31 Rf	0.3 AU	666.2 AU	100.00 %	Telmisartan*
2	0.63 Rf	1.4 AU	0.65 Rf	77.9 AU	100.00 %	0.67 Rf	0.3 AU	799.2 AU	100.00 %	Chlorthalidone *

FIGURE-42

CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC

REPEATABILITY -3

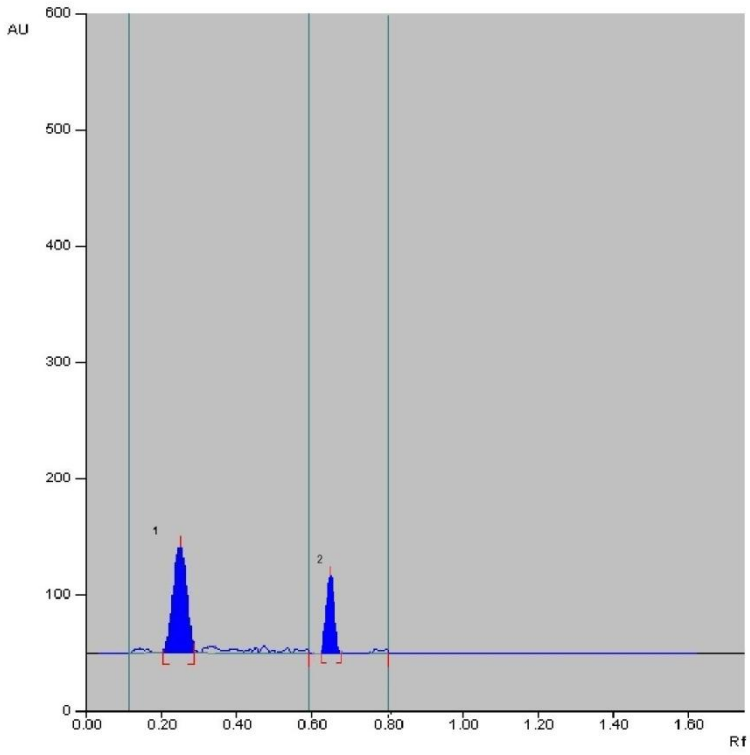


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.24 Rf	6.9 AU	0.28 Rf	19.8 AU	100.00 %	0.34 Rf	0.6 AU	673.8 AU	100.00 %	Telmisartan*
2	0.62 Rf	2.5 AU	0.64 Rf	110.9 AU	100.00 %	0.69 Rf	10.0 AU	799.2 AU	100.00 %	Chlorthalidone*

FIGURE-43

CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC

REPEATABILITY -4

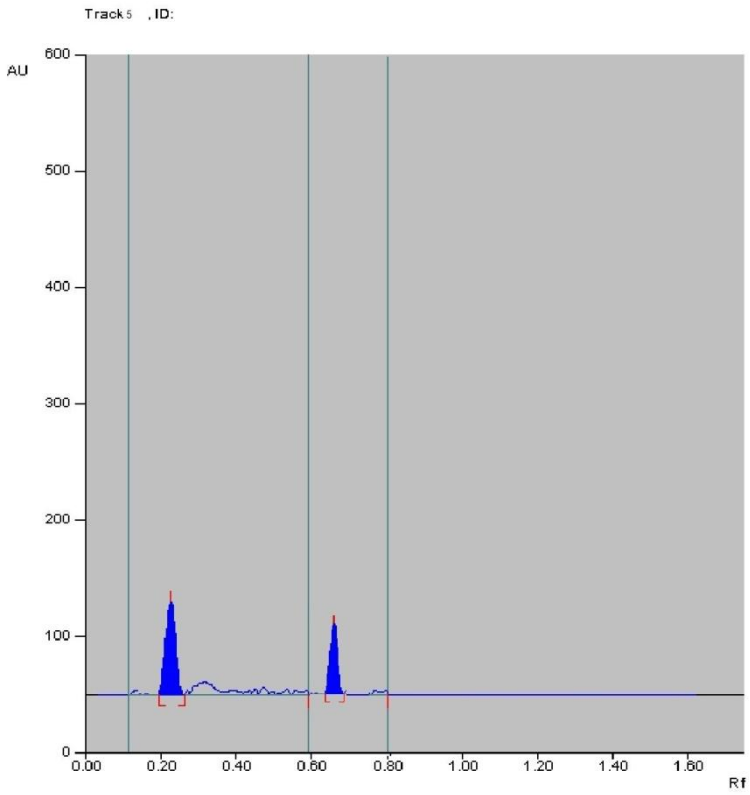


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.26 Rf	6.8 AU	0.29 Rf	29.2 AU	100.00 %	0.34 Rf	1.7 AU	677.4 AU	100.00 %	Telmisartan *
2	0.62 Rf	0.2 AU	0.65 Rf	61.4 AU	100.00 %	0.69 Rf	0.7 AU	793.1 AU	100.00 %	Chlorthalidone *

FIGURE-44

CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC

REPEATABILITY -5

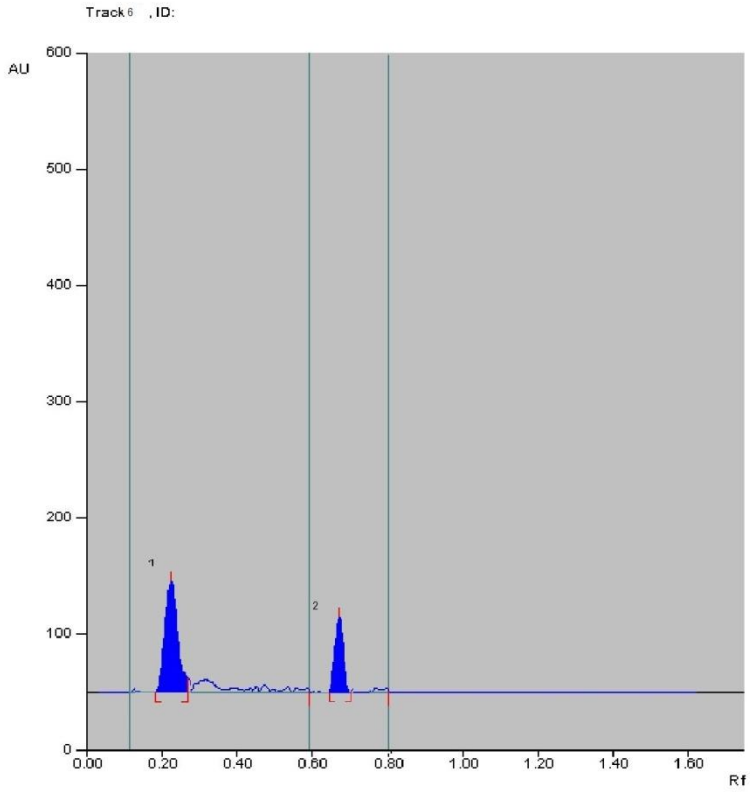


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.20 Rf	8.1 AU	0.27 Rf	122.2 AU	100.00 %	0.36 Rf	2.2 AU	675.4 AU	100.00 %	Telmisartan *
2	0.63 Rf	0.3 AU	0.65 Rf	108.9 AU	100.00 %	0.67 Rf	0.0 AU	785.7 AU	100.00 %	Chlorthalidone *

FIGURE-45

CHROMATAGRAM FOR FORMULATION (ERITEL - CH 40) ANALYSIS BY HPTLC

REPEATABILITY -6

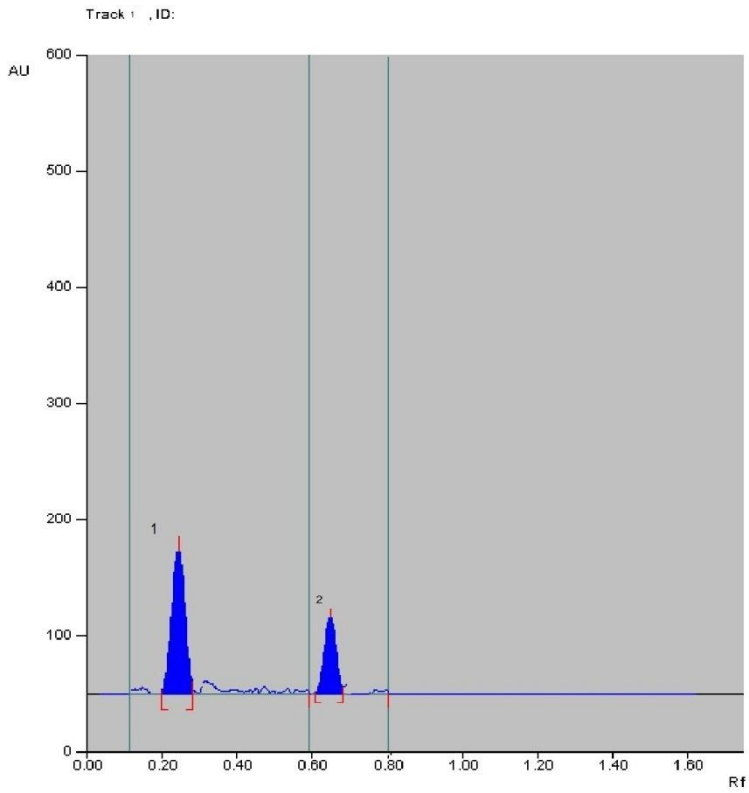


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.22 Rf	6.0 AU	0.28 Rf	98.4 AU	100.00 %	0.33 Rf	8.0 AU	666.5 AU	100.00 %	Telmisartan *
2	0.62 Rf	0.1 AU	0.65 Rf	35.1 AU	100.00 %	0.68 Rf	0.0 AU	798.2 AU	100.00 %	Chlorthalidone *

FIGURE-46

CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40) BY HPTLC

RECOVERY 1

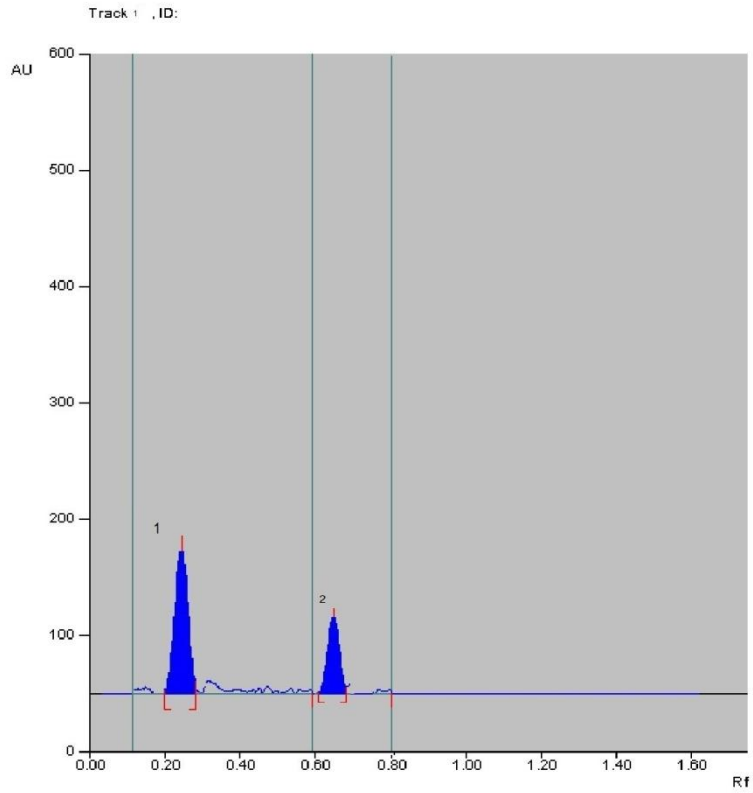


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.21 Rf	5.4 AU	0.29 Rf	52.8 AU	100.00 %	0.31 Rf	0.5 AU	1209.4 AU	100.00 %	Telmisartan *
2	0.64 Rf	0.2 AU	0.66 Rf	140.7 AU	100.00 %	0.69 Rf	18.4 AU	1374.6 AU	100.00 %	Chlorthalidone*

FIGURE-47

CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40) BY HPTLC

RECOVERY - 2

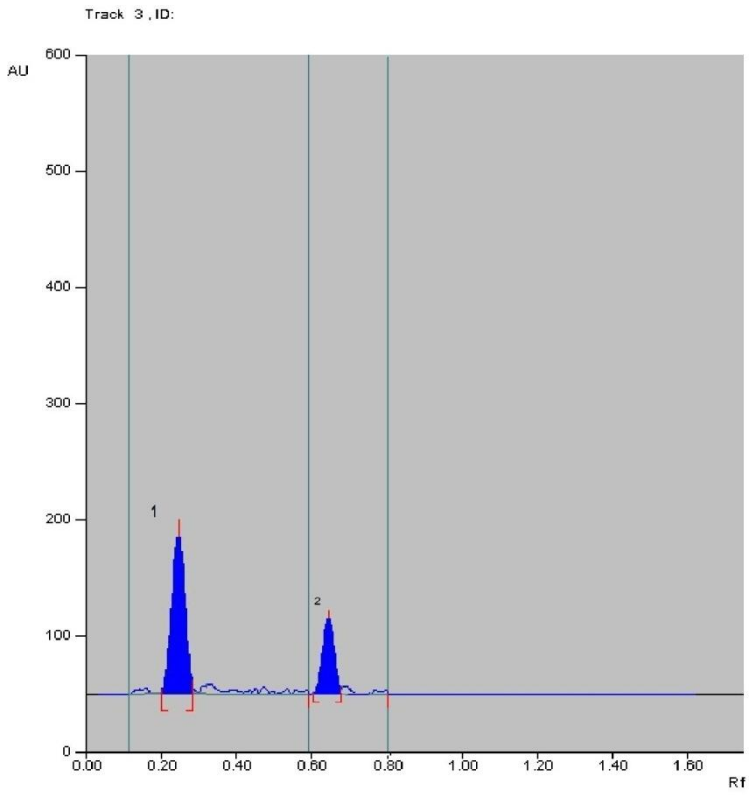


Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.26 Rf	6.8 AU	0.29 Rf	29.2 AU	100.00 %	0.34 Rf	1.7 AU	1344.8 AU	100.00 %	Telmisartan *
2	0.62 Rf	0.2 AU	0.65 Rf	61.4 AU	100.00 %	0.69 Rf	0.7 AU	1514.4 AU	100.00 %	Chlorthalidone *

FIGURE-48

CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40) BY HPTLC

RECOVERY – 3



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.20 Rf	8.1 AU	0.27 Rf	122.2 AU	100.00 %	0.36 Rf	2.2 AU	1478.3 AU	100.00 %	Telmisartan *
2	0.63 Rf	0.3 AU	0.65 Rf	108.9 AU	100.00 %	0.67 Rf	0.0 AU	1653.4 AU	100.00 %	Chlorthalidone *

TABLES

Table 1

SOLUBILITY PROFILE OF TELMISARTAN

S.NO	SOLVENT	EXTENT OF SOLUBILTY	CATEGORY
1.	Distilled water	More than 100 ml	Practically insoluble
2.	0.1 M NaOH	10 mg in 0.25ml	Soluble
3.	0.1 M HCL	10 mg in 0.2 ml	Soluble
4.	Acetonitrile	10 mg in 70 ml	Very slightly soluble
5.	Acetone	10 mg in 20 ml	Very slightly soluble
6.	Benzene	More than 100 ml	Insoluble
7.	Chloroform	10 mg in 7 ml	Slightly soluble
8.	Carbon tetra chloride	More than 100 ml	Insoluble
9.	Cyclohexane	More than 100 ml	Insoluble
10.	Dimethyl formamide	10 mg in 0.85 ml	Sparingly soluble
11.	Diethylamine	10 mg in 0.06 ml	Practically insoluble
12.	Dichloromethane	10 mg in 0.550 ml	Freely soluble
13.	Ethanol	10 mg in 30 ml	Very slightly soluble
14.	Isopropyl alcohol	More than 100 ml	Insoluble
15.	Methanol	10 mg in 8 ml	Slightly soluble
16.	N-Butanol	10 mg in 60 ml	Very slightly soluble
17.	N-Hexane	More than 100 ml	Practically insoluble
18.	Petrolium Spirit	More than 100 ml	Practically insoluble
19.	Pyridine	10 mg in 0.3 ml	Soluble
20.	Acid Phathale buffer pH3.0	More than 100 ml	Practically insoluble
21.	Acid Phathale buffer pH4.0	More than 100 ml	Practically insoluble
22.	Phosphate buffer pH 7.0	More than 100 ml	Practically insoluble
23	Phosphate buffer pH 8.0	More than 100 ml	Practically insoluble
24	Alkaline borate buffer pH 8.0	More than100 ml	Practically insoluble
25	Alkaline borate buffer pH 9.0	More than 100 ml	Practically insoluble
26	Alkaline borate buffer pH 10	More than 100ml	Practically insoluble

Table 2

SOLUBILITY PROFILE OF CHLORTHALIDONE

S.NO	SOLVENT	EXTENT OF SOLUBILTY	CATEGORY
1.	Distilled water	More than 100 ml	Practically insoluble
2.	0.1 M NaOH	10 mg in 0.35ml	Sparingly soluble
3.	0.1 M HCL	More than 100 ml	Insoluble
4.	Acetonitrile	10 mg in 3 ml	Slightly soluble
5.	Acetone	10 mg in 0.06 ml	Freely soluble
6.	Benzene	More than 100 ml	Insoluble
7.	Chloroform	More than 7 ml	Slightly soluble
8.	Carbon tetra chloride	More than 100 ml	Insoluble
9.	Cyclohexane	More than 100 ml	Insoluble
10.	Dimethyl formamide	10 mg in 0.850 ml	Freely soluble
11.	Diethylamine	10 mg in 0.06 ml	Freely soluble
12.	Dichloromethane	10 mg in 0.550 ml	Sparingly soluble
13.	Ethanol	10 mg in 0.6 ml	Sparingly soluble
14.	Isopropyl alcohol	10 mg in 20 ml	Very slightly soluble
15.	Methanol	10 mg in 0.08 ml	Freely soluble
16.	N-butanol	10 mg in 8 ml	Slightly soluble
17.	N-Hexane	More than 100 ml	Practically insoluble
18.	Petroleum Spirit	More than 100 ml	Practically insoluble
19.	Pyridine	10 mg in 0.08 ml	Freely soluble
20.	Acid Phathale buffer pH3.0	More than 100 ml	Practically insoluble
21.	Acid Phathale buffer pH4.0	More than 100 ml	Practically insoluble
22.	Phosphate buffer pH 7.0	More than 100 ml	Practically insoluble
23	Phosphate buffer pH 8.0	More than 100 ml	Practically insoluble
24	Alkaline borate buffer pH 8.0	More than100 ml	Practically insoluble
25	Alkaline borate buffer pH 9.0	More than 100 ml	Practically insoluble
26	Alkaline borate buffer pH 10	More than 100ml	Practically insoluble

Table 3

OPTICAL PARAMETER OF TELMISARTAN

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

PARAMETERS	**AT 311 nm***
Beers law limit (µg/ ml)	5 – 40
Molar absorptivity (L $mol^{-1}cm^{-1}$)	932.003
Sandell's sensitivity (µg/cm^2/0.001 A.U)	0.5733
Correlation coefficient (r)	0.9999
Regression equation (Y=mx+c)	Y = 0.001744x +0.000667
Slope (m)	0.001744
Intercept (c)	0.0006672
LOD (µg/ ml)	0.037716
LOQ (µg/ ml)	0.11429
Standard Error	0.0005218

(*Mean of six observations)

Table 4

OPTICAL PARAMETER OF CHLORTHALIDONE

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

PARAMETERS	**AT 251 nm***
Beers law limit (µg/ ml)	2 – 24
Molar absorptivity (L $mol^{-1}cm^{-1}$)	345.923
Sandell's sensitivity (µg/cm^2/0.001 A.U)	0.981964
Correlation coefficient (r)	0.9999
Regression equation (Y=mx+c)	Y = 0.0010184x+ (-) 0.0000266
Slope (m)	0.0010184
Intercept (c)	0.0000266
LOD (µg/ ml)	0.03393
LOQ (µg/ ml)	0.10283
Standard Error	0.0001267

(*Mean of six observations)

Table 5

ANALYSIS OF SYNTHETIC MIXTURE

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

Drug	Theoretical concentration	Experimental value (µg/ml)*	Percentage Obtained*	Mean	S.D	% RSD	SE
TEL	5	5.0255	100.50	100.77	0.7866	0.7806	0.0160
	10	10.0035	100.03				
	15	15.1631	101.08				
	20	19.9596	99.80				
	25	25.3390	101.35				
	30	30.6228	102.07				
	35	35.2091	100.60				
CHL	2	1.9704	98.51	98.51	0.6856	0.6959	0.01399
	4	3.9014	97.54				
	8	7.8125	97.62				
	12	11.8546	98.76				
	16	15.8149	98.84				
	20	19.7915	98.95				
	24	23.8499	99.36				

(* Mean of six observations)

Table 6

QUANTIFICATION OF FORMULATION (ERITEL - CH 40)
(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

Drug	Labelled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%)	S.D	% RSD	SE	CI
TEL	40 40 40 40 40 40	40.7522 40.0355 40.1550 39.8444 40.0594 39.9639	101.88 100.08 100.38 99.61 100.15 99.91	100.33	0.7993	0.7966	0.0222	99.01 to 101.64
CHL	12.5 12.5 12.5 12.5 12.5 12.5	12.5337 12.5746 12.4927 12.4927 12.4270 12.4928	100.26 100.60 99.94 99.94 99.98 99.94	99.99	0.4373	0.4304	0.0121	99.27 to 100.70

(*Mean of six observations)

Table 7

INTRADAY AND INTER DAY ANALYSIS OF FORMULATION

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

Drug	Amount labeled (mg/tab)	Percentage Obtained*		SD		%RSD	
		Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
TEL	40	101.29	101.94	0.9540	1.0209	0.9418	1.0015
	40	99.73	99.43	0.7260	0.6168	0.7280	0.6203
	40	100.03	101.59	1.1191	0.9056	1.1186	0.9056
CHL	12.5	99.95	100.28	1.4785	1.0209	1.4792	1.0093
	12.5	100.12	99.43	1.1471	1.4661	1.1457	1.4667
	12.5	100.29	101.59	1.0095	1.0662	1.0067	1.0597

(*Mean of six observations)

Table 8

RUGGEDNESS STUDY

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

PARAMETER	PERCENTAGE AMOUNT FOUND		SD		%RSD	
	TEL	CHL	TEL	CHL	TEL	CHL
DIFFERENT ANALYST	99.80	99.93	0.7627	0.42763	0.7643	0.4279

(*Mean of six observations)

Table 9

RECOVERY ANALYSIS OF FORMULATION (ERITEL – CH 40)

(FIRST ORDER DERIVATIVE SPECTROSCOPY)

Drug	Amount Present (µg/tab)	Amount Added (µg/tab)*	Amount Estimated (µg/tab)*	Amount Recovered (µg/tab)*	Percentage Recovered*	SD	%RSD	SE
TEL	16.1567 16.1567 16.1567	13.2549 16.8176 19.6671	29.2672 33.1006 36.3588	13.0105 16.9439 20.2021	99.13 100.75 102.72	1.7978	1.7823	0.1997
				Mean	100.62	CI = 100.26 to 102.77		
CHL	4.9977 4.9977 4.9977	4.2288 4.9817 5.9637	9.2253 9.9781 11.0855	4.2276 4.9804 6.0551	99.98 99.97 101.52	1.1207	1.1152	0.01384
				Mean	100.49	CI = 98.85 to 102.87		

(* Mean of three observation

TABLE – 10

SYSTEM SUITABILITY PARAMETR

PARAMETERS	TELMISARTAN	CHLORTHALIDONE
Retention time	2.915	4.637
Tailing factor	1.176	1.133
Asymmetrical factor	1.146	1.119
Theoretical plates	3826	2130
Capacity factor	1.2083	2.5128
Resolution	Between TEL and CHL 9.187	

Table 11

OPTICAL PARAMETER OF TELMISARTAN AND CHLORTHALIDONE BY RP-HPLC

PARAMETERS	TELMISARTAN*	CHLORTHALIDONE*
λ_{max} (nm)	229nm	229nm
Beers law limit (μg mL^{-1})	5-25	2-16
Correlation coefficient (r)	0.9994	0.9996
Régression équation (y=mx+c)	Y= (83176)x+6710.61	Y=(75502)x-(6399.85)
Slope (m)	83176	75502
Intercept (c)	6710.61	6399.85
LOD (μg mL^{-1})	0.17426	0.101042
LOQ (μg mL^{-1})	0.5280	0.30621
Standard Error	28090.69	11229.25

FIGURE -12

QUANTIFICATION OF FORMULATION (ERITEL-CH 40)

BY RP – HPLC

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg)	Percentage Obtained *	Average (%)	S.D	% R.S.D.	S.E.
TEL	1	40	40.7148	101.79	101.63	0.7025	0.69122	0.0195
	2	40	40.4804	101.20				
	3	40	40.5334	101.33				
	4	40	40.4825	101.20				
	5	40	40.5081	101.27				
	6	40	40.1978	102.99				
					CI=100.89 to 102.36			
CHL	1	12.5	12.6410	101.12	101.46	0.4449	0.4379	0.01236
	2	12.5	12.5592	100.47				
	3	12.5	12.7512	102.00				
	4	12.5	12.6016	100.81				
	5	12.5	12.6897	101.59				
	6	12.5	12.7360	101.51				
					CI=100.72 to 102.19			

* Mean of Six Observations

TABLE – 13

RECOVERY ANALYSIS OF FORMULATION (ERITEL-CH 40) BY RP – HPLC

Drug	Sample No.	Amount present (µg/ml)	Amount added (µg/ml)	Amount estimated* (µg/ml)	Amount recovered (µg/ml)	% Recovery*	S.D	% R.S.D	S.E.
TEL	1	8.1306	6.4	14.5392	6.4087	100.14	1.6042	1.6019	0.1782
	2	8.1306	8	16.0862	7.9556	99.45	0.6238	0.6273	0.0693
	3	8.1306	9.6	17.9000	9.7695	101.76	0.5861	0.5760	0.0651
					Mean	100.45			
CHL	1	2.5326	2	4.5727	2.0401	102.01	0.4501	0.4412	0.0500
	2	2.5326	2.5	5.0097	2.4771	99.08	0.2775	0.2801	0.0308
	3	2.5326	3	5.5860	3.0542	101.80	0.3592	0.3528	0.0399
					Mean	100.96			

* Mean of Six Observations

TABLE- 14

OPTICAL CHARACTERISTICS OF TELMISARTAN AND CHLORTHALIDONE BY HPTLC

PARAMETERS	TELMISARTAN	CHLORTHALIDONE
Detection wavelength	229 nm	229 nm
Linearity range (ng/μl)	100 – 800	50 – 400
Correlation Coefficient (r)	0.9992	0.9989
Regression Equation (y=mx+c)	Y=2.1182 +(-16.9111)	Y = 7.06113x+80.8474
Slope (m)	2.1182	7.06113
Intercept (c)	-16.9111	80.8474
LOD (ng/μl)	0.22446	0.5206
LOQ (ng/μl)	0.68019	1.5775
Standard Error	2.47903	4.7374

TABLE – 15

QUANTIFICATION OF FORMULATION (ERITEL – CH 40)

BY HPTLC

Drug	Sample No.	Labelled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%) ± SD	% RSD.	SE	CI
TEL	1	40	40.8965	102.24	101.66 ± 0.7853	0.7725	0.0218	100.36 to 102.95
	2	40	40.3105	100.77				
	3	40	40.7179	101.77				
	4	40	40.9714	101.42				
	5	40	40.8532	102.13				
	6	40	40.2397	100.59				
CHL	1	12.5	12.7264	101.81	101.31 ± 0.8336	0.8228	0.02319	99.94 to 102.68
	2	12.5	12.7538	102.05				
	3	12.5	12.6086	101.73				
	4	12.5	12.7166	100.86				
	5	12.5	12.4776	99.82				
	6	12.5	12.6989	101.59				

TEL = Telmisartan, CHL=Chlorthalidone,

*Mean of six observations

CI[@] = confidence interval (95 %)

TABLE – 16

RECOVERY ANALYSIS OF FORMULATION (ERITEL-CH 40) BY RP – HPTLC

Drug	Sample No.	Amount present (ng/µl)	Amount added (ng/µl	Amount estimated* (ng/µl	Amount recovered* (ng/µl	% Recovery	S.D	% R.S.D	S.E.
TEL	1	325.30	256	578.78	253.43	98.99	0.1201	0.1212	0.0133
	2	325.30	320	642.83	317.53	99.23			
	3	325.30	384	705.86	380.56	99.10			
					Mean	99.10	CI = 98.90 to 99.29		
CHL	1	101.31	80	183.22	81.9116	102.38	0.6319	0.6034	0.0682
	2	101.31	100	202.96	101.6535	101.65			
	3	101.31	120	222.65	121.3388	101.16			
					Mean	101.73	CI = 100.68 to 102.77		

BIBLIOGRAPHY

1. Ajit Pandey, Swarkar H, Mukesh Singh, Kashyap P and Priyanka Ghosh. UV-Spectrophotometric method for estimation of Telmisartan in bulk and tablet dosage form , *Int.J.ChemTech Res.*, **2011**, 3(2), 654-660.
2. Asha B.Thomas, Sheetal N.Jagdale, Shweta B.Dighe and Rabindra K.Nanda. Simultaneous spectrophotometric estimation of amlodipine besylate and telmisartan in tablet dosage form, *Int.J.ChemTech Res.*, **2010,** 2(2), 1334-1341.
3. Akiful Haque.M, Nivedita.G, Prashanth Kumar.K, Pradeep Kumar.T, Hasan Amrohi.S and Prakash.V.diwan. Simultaneous estimation of atenolol and chlorthalidone as bulk and in tablet dosage form using UV-Spectrophotometry, *IOSRJPBS*, **2012**,1(4) , 20-23.
4. Anonymous. Clarke's Analysis of Drugs and poison, 3rd Edition, The Pharmaceutical Press, division of Royal Pharmaceutical Society of Great Britain, London, **2004,**796 and 1601.
5. Anonymous. Martindale, The Extra Pharmacopoeia, 30th Edition The Pharmaceutical Press, London, **1993**,813-1.
6. Anonymous. The Pharmaceutical Codex, Principles & Practice of Pharmaceuticals, 12th Edition The Pharmaceutical Press, London, **2009**,802.
7. Anonymous. The British Pharmacopeia, Volume I, II and IV, International Edition, Office of the British Pharmacopeia Commission, London **2009**,
8. Anonymous. The Merck Index, An Encyclopedia of Chemicals, Drugs and Biologicals, 14th Edition, Merck Research Laboratories, a division of Merck and Co. Inc., NJ, USA, **2006,** 2193 and 9129.
9. Anonymous. The United States Pharmacopeia. The United States Pharmacopoeial Convention, Inc., Rock Ville, MD, USA, **2009,** 2246.
10. Bankey S, Tapadiya G.G, Saboo S.S, Bindaiya S, Deepti Jain and Khadbadi S.S. Simultaneous determination of ramipril, hydrochlorthizide and telmisartan by spectrophotometry, *Int.J.ChemTech Res.*, **2009,** 1(2), 183-188.
11. Beckett A. H. and Stenlake J. B. Practical Pharmaceutical Chemistry, Volume II, 4th Edition, CBS Publishers and Distributors, New Delhi, India, **2007**, 143, 278-300.
12. Bhaskara Raju V and Lakshmana Rao. Novel HPLC method development and Validation and estimation of telmisartan in bulk and its pharmaceutical formulation, *Int.J.ChemTech Res.*, **2011**,3(4), 2001-2005.

13. Bhatia NM, Shinde HV, Bhatia MS, Chudhari PB and Ingale KB. Development and validation of spectrophotometric and ion pair chromatographic techniques for estimation of telmisartan and hydrochlorthiazide, *Ars Pharm,* **2010**, 51(3), 145-154.
14. Bolton S. Pharmaceutical Statistics, Practical and Clinical Applications, 2nd Edition, Marcel Dekker, Inc., New York **2004**, 157,176 and 210.
15. Brijesh Singh Patel D.K and Ghosh S.K. A reversed-Phase high performance liquid chromatographic method for determination of chlorthalidone in pharmaceutical formulation,Int.J.Pharm.Pharm.Sci., **2009**, 1(2), 24-29.
16. Code Q2A, Text on Validation of Analytical Procedures. ICH Harmonized Tripartite Guidelines, Geneva, Switzerland, **1994,** 1 – 5.
17. Code Q2B, Validation of Analytical Procedures; Methodology. ICH Harmonized Tripartite Guidelines, Geneva Switzerland, **1996,** 1 – 8.
18. Douglas A. Skoog. Principles of instrumental analysis, 5th Edition, Thomson Business Information India Pvt Ltd, New Delhi, **2006,** 1 – 18.
19. Elshanawane AA, Mostafa SM and Elgawish MS.Development and validation of a Reversed-phase high-performance liquid chromatographic method for the simultaneous determination of amiloride hydrochloride, atenolol, hydrochlorothiazide and chlorthalidone in their combined mixture, *J AOAC Int*., **2009**, 92(2), 404-409.
20. Faimida Jahan, Avnish Jain Sumeet Prachand and Arun Kumar Gupta. Simultaneous estimation of telmisartan and cilnidipine in solid dosage form, *Int.J.of Pharm & Research Sci.,* **2012,** 1(1), 32-42.
21. Fifeld F.W. and Kealey D. Principles and Practice of Analytical Chemistry, 5th edition, Blackwell Publishers, London, **2004,** 5.
22. Gupta S.C. and Kapoor V.K. Fundamentals of Mathematical Statistics, 9th edition, Sultan Chand and Sons, New Delhi, **1995**, 2.6, 3.2 - 3.28.
23. Gurudeep R. Chatwal and Sham K. Anand, Instrumental Methods of Chemical Analysis, 5th Revised Edition, Himalaya Publishing House, Mumbai, **2008,** 2.149 – 2.170, 2.566 – 2.568.
24. Hobart H.Willard, Lynne L.Meritt,Jr., John A.Dean and Frank A.Settle. Instrumental Methods of Analysis, 7th edition, CBS Publishers and Distributors, New Delhi, **1986**, 169.

25. Jat R.K, Sarma S,Chippa R.C, Singh Rambir and alam Imran. Quantitative estimation of telmisartan in bulk drug and tablet by UV spectroscopy, Int.J.Drug Res.Tech., **2012**, 2(3),268-272.
26. Kalyan Kumar B, Santhos Kumar T, Shravan Kumar A and Venkadeshwar Rao P. Development and validation of RP-HPLC method for simultaneous estimation of ramipril, telmisartan and Hydrochlorthiazide in Pharmaceutical dosage forms, *J. Pharm.Res.*,**2011**,4(10), 3306-3308.
27. Kondawar M.S, Kamble K.G, Raut K.S and Maharshi K.H. UV Spectrophotometric estimation of amlodipine besylate and telmisartan in bulk drug and dosage form by multiwavelegth analysis, *Int.J.ChemTech Res.*, **2011**,3(3), 1274-1278.
28. Kumar G.S , Ramya V, Sumanta, Mondal and Sai Pavan Kumar. Development and validation of RP-HPLC method for simultaneous estimation of aenolol and chlorthalidone from pharmaceutical formulation, *IRJP*, **2012**, 3(10), 215-219.
29. Kumbhar S.T, Chougule G.K, Gajeli G.B,Tegeli V.S, Thorat Y.S and Shivsharan U.S. Visible Spectrophotometric determination of telmisartan from urine, *IJPSR*, **2011**, 2(5), 1254-1258.
30. Kurade VP, Pai MG and Gude R RP-HPLC estimation of ramipril and telmisartan in tablets, *Indian J. Pharm. Sci.*, **2009,** 71(2),148-151.
31. Lakshmi KS and Lakshmi S. Design and optimizationof a chemometric-Assisted spectrophotometric determination of telmisartan and hydrochlorothiazide in pharmaceutical dosage form, *J. Young Pharm.*, **2010,** 2(1), 85-89.
32. Leena R.Bhat, Rahul K.Godge, Asfak T.Vora and Mrinalini C.Damle. Validated RP-HPLC method for simultaneous determination of telmisartan and hydrochorohiazide in pharmaceutical formulation, J.Liq.Chromatogr.&Related Tech., **2007**, 30(20), 3059-3067.
33. Luz Luis M, Jose M. Garcia, Francisco Jimenez, Ana I.Jimenez and Jugan J.Arias. Simultaneous Determination of Chlorthalidone and Spironolactone with univariate and multivariate calibration wavelength range selection, *J. AOAC Int.*, **1999,** 82(5), 1054-1063.
34. Lloyd R .Snyder, Joseph J. Kirkland and Joseph L. Glajch. Practical HPLC Method Development, 2nd Edition, Wiley Interscience Publication, John Wiley and Sons Inc., **1994,** 101 – 140, 317 – 339 and 653 – 660.

35. Madhu Babu Kasimala and Bikshal Babu Kasimala.Reverse Phase-HPLC method development and validation for the simultaneous estimation of Azilsartan medoximil and Chlorthalidone in pharmaceutical dosage forms, *jamonline,* **2012,** 2(1), 117-126.
36. Mayur Modi, Rikin Shah and Mashru R.C. Development and validation of spectrophotometric methods for simultaneous estimation of metoprolol succinate and telmisartan in combined pharmaceutical formulation, *IJPSR*, **2012**, 3(5), 1348-1354.
37. Mendham J., Denny R.C., Jeffery G.H. and Thomas. Vogel's text book of Quantitative Chemical Analysis, 5th Edition, Longman publishers, UK, **1994**, 10 – 11.
38. Mhaske R.A, Garole D.J, Mhaske A.A and Sahasrabudhe. RP-HPLC method for simultaneous determination of amlodipine besylate, valsartan, telmisartan, Hydrochlorothiazide and chlorthalidone –application to commercially available drug products, *IJPSR*, **2012**, 3(1), 141-149.
39. Mhaske R.A, Sahasrabudhe, Mhaske A.A and Garole D.J. RP-HPLC method for simultaneous determination of atrovastatin calcium olmisartan medoxomil, Candesartan, Hydrochlorothiazide and chlorthalidone –application to commercially available drug products, *IJPSR*, **2012**,3(3),793-801.
40. Nada.S.Abdelwahab, Determination of atenolol ,Chlorthalidone and heir degradation product by TLC-densitometric and chemometric method with application of model updating, *Anal.Methods*, **2010**, 2,1994-2001 .
41. Niranjan D. Chivate, Siddharth M.Patil, Jagadish K. Saboji and Anuraha N.Chivate. Development of UV spectrophotometric method for estimation and validation of telmisartan as a pure API, *J. Pharm.Res.,* **2012**, 5(6), 3331-3333.
42. PalledMS, Chatter M, Rajesh PMN and Bhat AR. Difference Spectrophotometric determination of telmisartan in tablet dosage forms, *Indian J. Pharm. Sci.,* **2006**, 68(5), 685-686.
43. Patil U.P Gandhi S.V, Sengar M.R and Rajmane V.S. Simultaneous determination of atrovastatin calcium and telmisartan in tablet dosage form by spectrophotometry, *Int.J. ChemTech Res.,* **2009**, 1(4), 970-973.
44. Popt B. Mohite, Ramdas b.Pandhara and Vaidhun H.Bhaskar. Simultaneous estimation of ramipril and telmisartan in tablet dosage form by spectrophotometry, *Eurasian J.Anal.Chem.,* **2010,** 51(1), 89-94.
45. Pratap Y. Pawar, Manish A. Raskar, Swati U.Kalure, and Reshma B.kulkarni. Simultaneous spectrophotometric estimation of amlodipine besylate and telmisartan in tablet dosage form, *Der Pharma Chemica*, **2012**, 4(2), 72-730.

46. Ramesh L,Sawant, Manish A.Rakar Raihan Ahemed and Sameer Pawar. Validated spectrophotometric methods for simultaneous estimation of telmisartan and indapamide in pharmaceutical dosage form, *Der Pharma chemica*, **2012**, 4(2), 633-638.
47. Ridhdhi S Sinojiya, Vasant Khasia, Bhaven J Patel and Amish A Dangi. Development and validation of RP-HPLC methods for the simultaneous determination of telmisartan, amlodipine Besylate and Hydrochlorthiazide in a tablet dosage form, *J.Pharm.Res.,* **2012**, 5(8), 4154-4157.
48. Robert D.Braun, Introduction to Instrumental Analysis, Pharma Book Syndicate, Hyderabad, **2006,**136.
49. Sagar Tatane. Development of UV-Spectrophotometric method of telmisartan in tablet formulation, *JAPHR*, **2011,** (1), 23-26.
50. Santaji Nalwade,Vangala Ranga Reddy, Dantu Durga Rao and Inabathina Koteswara Rao. Rapid simultaneous determination of telmisartan, amlodipine besylate and hydrochlorothiazide in a combined poly pill dosage form by stability- indicating ultra performance liquid chromatography, *Sci. Pharm.,* **2011**, 79, 69-84.
51. Santosh V.Gandhi, Padmanabh B.Deshpande, Varun Godbole, Pankaj Jagdale, Sachin Khiste and Sayali Kadukar. A Validated reverse phase HPLC method for the simultaneous determination of telmisartan and ramiprilas bulk drug and in tablet dosage form, *J. Chem. Bio. Phy. Sci.,* **2011**, 1(2), 283-288.
52. Sethi P.D. HPTLC Quantitative Analysis of Pharmaceutical Formulations, 1st Edition, CBS publishers and Distributors, New Delhi, **1996,** 3 – 62.
53. Sharma B.K. Instrumental Methods of Chemical Analysis, 25th edition, Goel Publishing House, Meerut, **2006,** S 68 – S 192.
54. Subhakar Nandipati, Krishna Reddy V and Ravindranadh Reddy. Development and validation of RP-HPLC Method for estimation of telmisartan in bulk and tablet dosage form, *Int.Res. J. Pharm.App. Sci.,* **2012**, 2(3), 39-43.
55. Sunil Jawla , Jayalakshmi K, Krishnamurthy T and Kumar Y. Development and validation of simultaneous HPLC method for estimation of telmisartan and ramipril in pharmaceutical formulation, *Int.J.ChemTech Res.,* **2010,** 2(2), 1625-1633.
56. Vijayamirtharaj R, Ramesh J, Jayalakshmi B and Hanas Bin Hashim. Development and validation of RP HPLC method for the simultaneous estimation of tellmisartan and atrovastatin calcium intablet dosage forms, *Pharmacie Globale (IJCP)*, **2010**, 4(3), 1-4.

57. Vijay Kumar G, Murthy TEGK, and Sambasiva Rao KRS. Validated RP-HPLC method for the estimation of telmisartan in serum samples, *IJRPC*, **2011**, 1(3), 703-706.
58. Yogesh Gupta and Alankar Shrivastava. Isocratic RP-HPLC-UV method development and validation for the simulataneous estimation of ramipril and telmisartan in tablet dosage form, *Asian J.Pharm.Clin.Res.*, **2009**, 2(4), 104-111.